Evaluation of Biomarkers for Testicular Toxicity

Naomi D. Elkin

BSc (hons) Biochemistry, University of Bristol

MRC Human Reproductive Sciences Unit,
Centre for Reproductive Biology, University of Edinburgh,
Queen's Medical Research Institute,
47 Little France Crescent, Edinburgh, EH16 4TJ

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Abstract

Non-clinical safety assessment is essential during the drug development process in the pharmaceutical industry, and involves numerous, detailed in vitro and in vivo toxicology tests (general, reproductive and genetic), and safety pharmacology studies. The testis is a common organ for adverse drug effects leading to attrition of potential compounds. It would, therefore, be useful to detect testicular toxicity as early as possible in the drug development process. Histopathology is the standard method for assessing testis toxicity, but a biomarker for 'early warning' detection of testicular toxicity would be far more useful in non-clinical toxicology studies. The aim of this thesis was to evaluate the feasibility of this approach.

It is thought that proteins can leak from seminiferous tubules into testicular interstitial fluid following testicular damage, due to either loss of integrity of the blood-testis barrier (BTB) or germ cell damage. A potential biomarker protein could, therefore, leak out of seminiferous tubules into interstitial fluid and then into blood following toxicological insult to the testis. A suitable biomarker protein must be testis specific, abundant, and not normally be present in blood. It may also need to have a low molecular weight. To investigate if proteins do leak out of seminiferous tubules following testicular damage, three known testicular toxicants which affect different aspects of the testis were used; cadmium chloride causes disruption to the blood-testis barrier and spermatogenesis, methoxyacetic acid (MAA) specifically causes a loss of pachytene spermatocytes, and 1,3-dinitrobenzene (DNB) causes Sertoli cell vacuolation and subsequent germ cell disruption. Adult male Wistar rats were treated with various doses of these toxicants to give mild and moderate responses. Samples were collected 24 hours later. Testicular damage was investigated by immunohistochemistry for well-known germ cell markers (DAZL, VASA) and using a general antibody to seminiferous tubule proteins. The integrity of the BTB was evaluated using immunofluorescent co-localisation of occludin, ZO-1, claudin-11, N-cadherin and β-catenin, and a biotin tracer. Protein leakage was investigated using analysis of interstitial fluid samples by 1D gel electrophoresis and

staining with Coomassie-based dye or Western blotting for germ cell proteins and with the general antibody to seminiferous tubule proteins.

Protein leakage from seminiferous tubules into interstitial fluid was observed with high dose cadmium chloride treatment. This was coincident with a loss of integrity of the BTB. No leakage was observed with MAA treatment which caused a specific loss of pachytene spermatocytes, or DNB which caused Sertoli cell vacuolation. With both treatments the BTB did not appear to be damaged suggesting that protein leakage occurs only following loss of integrity of the BTB. This was further investigated using treatments reported to specifically disrupt the BTB, namely intra-testicular administration of glycerol or transforming growth factor-β3, with samples collected 48 hours later. The damage caused was very localised, although BTB disruption with glycerol treatment caused some protein leakage. The presence of germ cell proteins in interstitial fluid samples before and after the development of the BTB during normal development was also evaluated, although most proteins of interest were not expressed in germ cells of the immature testis before BTB formation.

Finally, five potential biomarker candidate proteins (ADAM3, Calpastatin, DAZL, FABP9, VASA) were selected and investigated using samples from the testicular toxicant studies. Smaller molecular weight proteins were thought to be more likely to leak out of seminiferous tubules, however, VASA, a large molecular protein (76kDa) was shown to leak into interstitial fluid following high dose cadmium chloride treatment. However, FABP9 (low molecular weight) was found to be the most promising biomarker for loss of BTB integrity.

The results suggest that a biomarker could only be detected if there is a loss of integrity of the BTB and severe disruption of spermatogenesis, thus conferring no real advantage over present histopathology-based toxicity evaluations. Therefore, an automated immunohistochemistry and image analysis method was investigated as a refined method for detection of testicular toxicity at the end of a toxicology study, and shown to have promise.

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Declaration

The studies described in this thesis were the unaided work of the author except where acknowledgement is made by reference. No part of this work has been previously accepted for, or is currently being submitted in candidature for another degree.

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Blood-Testis Barrier and Leakage of Proteins from Seminiferous Tubules in the Rat.

Miniposter presentation at the European Testis Workshop, Naantali, Finland, May 2008:
Cadmium chloride disrupts the Blood-Testis Barrier and causes Leakage of Proteins from Seminiferous Tubules in Adult Rats.

Poster presentation at the British Andrology Society/Asociación Espanola de Reproducción Animal International Congress, Gijon, Spain, October 2008:
Protein Leakage from Seminiferous Tubules following administration of Testicular Toxicants to Adult Male Rats.

Poster presentation at the Molecular Andrology International Workshop, Giessen, Germany, May 2009:
Protein Leakage from Germ Cells into Interstitial Fluid following administration of Testicular Toxicants to Adult Male Rats.

Abbreviations

Abbreviation	Definition
2-ME	2-methoxyethanol (same as EGME)
3Rs	Reduction, Replacement and Refinement of the use of animals in research
3β-HSD	3β-hydroxysteroid dehydrogenase
ABP	Androgen Binding Protein
ABPI	Association of the British Pharmaceutical Industry
ADAM3	A Disintegrin and Metalloprotease Domain 3
ANOVA	Analysis of Variance
AR	Androgen Receptor
ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumin
BTB	Blood-Testis Barrier
cAMP	cyclic Adenosine Monophosphate
$CdCl_2$	Cadmium chloride
cDNA	copy DNA
C_t	Threshold Cycle
CV	Cardiovascular
DAB	3,3'-diaminobenzidinetrahydrochloride
DAZL	Deleted in Azoospermia-Like
DBP	di(*n*-butyl) phthalate
DIG	Digoxigenin
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
DNB	1,3-dinitrobenzene
EDS	Ethane dimethanesulphonate
EGME	Ethylene glycol monomethyl ether (same as 2-ME)
ELISA	Enzyme-linked Immunosorbent Assay

ES	Ectoplasmic Specialisations
FABP9	Fatty Acid Binding Protein 9
FACTT	Fluorescent Amplification Catalysed by T7 Polymerase Technique
FDA	U.S. Food and Drug Administration
FSH	Follicle Stimulating Hormone
FTIH	First Time in Human
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GLP	Good Laboratory Practise
GSK	GlaxoSmithKline
HD	2,5-hexanedione
HRP	Horseradish Peroxidase
i.p.	Intraperitoneal
ICH	International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use
IF	Interstitial Fluid
Ig	Immunoglobulin
Insl3	Insulin like factor 3
JAM	Junction Adhesion Molecule
JNK	c-Jun N-terminal kinase
kDa	kilo Dalton
LDH-C4	Lactate Dehydrogenase C4
LH	Lutenising Hormone
LSAB	Labelled Streptavidin-Biotin
MAA	Methoxyacetic Acid
MAPK	Mitogen-activated protein kinase
MBP	Monobutyl phthalate
mRNA	messenger RNA
NB	Nitrobenzene
NNB	1-nitroso-3-nitrobenzene
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction

PoC	Proof of Concept
PV	Peripheral Vein Blood Plasma
Q-PCR	Quantitative PCR
RNA	Ribonucleic Acid
RT	Reverse Transcriptase
s	seconds
SEM	Standard Error of the Mean
SER	Smooth Endoplasmic Reticulum
SPAG5	Sperm Associated Antigen 5
ST	Seminiferous Tubule
StAR	Steroidogenic Acute Regulatory Protein
STCM	Seminiferous Tubule Conditioned Medium
TBS	Tris Buffered Saline
tCAST	Testis Specific Calpastatin
TGF-β3	Transforming Growth Factor-β3
TNF-α	Tumour Necrosis Factor-α
TUNEL	Terminal deoxynucleotidyl transferase-mediated (TdT) dUTP nick end labelling
ZO-1	Zonula occludens 1

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1 Introduction

An essential part of the drug development process in the pharmaceutical industry is an assessment of the potential adverse effects of a new drug compound. An evaluation of any potential adverse effects of the drug at the pharmacological dose must be carried out in relation to the benefits of the drug in order for it to be approved for use by the relevant regulatory bodies. The safety of potential drug compounds is evaluated in non-clinical and clinical studies. A range of non-clinical studies are carried out before first administration to humans in phase I clinical trials. Non-clinical assessments continue throughout the development process, with certain studies being carried out before administration of the drug to different groups of people in clinical trials. For example, potential drugs cannot be tested in clinical trials with women of child-bearing age, without a non-clinical evaluation of the potential effects of the drug on fertility and fetal development.

Non-clinical safety testing involves in vitro and in vivo toxicology tests (general, reproductive and genetic), and safety pharmacology studies. General regulatory toxicology studies involve administration of the potential drug compound to a range of animal species (usually one rodent (rat) and one non-rodent (dog)), for varying lengths of time, and with different doses. The toxicology of the potential drug is evaluated during these studies via monitoring of bodyweight and clinical observations, and at the end of these studies using histopathological analysis of tissue, and clinical chemistry. Any toxicology observed must be evaluated depending on the dose level (and how different it is to the pharmacological dose) and the effects observed. If the potential drug compound causes adverse effects at a pharmacologically relevant dose, depending on the risk:benefit ratio of the potential drug, it may not be developed further,

One of the most common target organs leading to cessation of development of a drug is the testis (GSK internal report). Early detection of toxicity leading to cessation of development is essential for reducing the number of animals used, and the cost of

drug development. Biomarkers are becoming increasingly important in bioscience, particularly in toxicology, and could have a role in early detection of testicular damage, and translation into the clinic for monitoring testicular damage. In toxicology studies, as well as early detection, a profile of testicular damage could also be obtained throughout the period of the study to provide an idea as to how the damage is caused, rather than just looking at the histopathology at one point in time; the end of the study.

The objective of this project is to investigate if sensitive biomarkers can be identified and measured as a means of providing an 'early warning' of testicular damage in non-clinical toxicology studies. In order to achieve this, candidate germ cell specific proteins will be identified, and the potential for their leakage from seminiferous tubules into interstitial fluid (and bloodstream) will be investigated in situations in which experimental damage to spermatogenesis has been induced.

2 Review of the Literature

2.1 Introduction

The objective of this thesis is to investigate the potential for identifying and developing biomarkers of testicular toxicity. Before this can be evaluated, knowledge of the testis is essential. An understanding of the safety assessment process in the pharmaceutical industry and why such biomarkers would be advantageous is also important. Both of these subjects are addressed in this chapter, as well as an introduction to well-studied testicular toxicants, and current methods of detection and previously investigated biomarkers for testicular toxicity.

2.2 Development of the Testis

In early embryonic development, the gonad is the same for males and females. The mesenchyme tissue from the genital ridge forms the basic structure of both male and female gonads (Johnson, 2007). Primordial germ cells, the precursor germ cells, develop from the coelemic epithelium in the hindgut mesentery (Brennan and Capel, 2004). They migrate to the genital ridge, guided by chemotactic agents (Johnson, 2007). Development of the testis is an active process, under the action of the Y-chromosome derived *Sry* gene (reviewed in Clarkson and Harley, 2002). *Sry* expression promotes differentiation of cells derived from the coelomic epithelium, the precursor Sertoli cells. These cells penetrate the mesenchyme and aggregate around the primordial germ cells, forming testis cords (Brennan and Capel, 2004). Cells also migrate from the mesenophric primordia into the developing gonad. These cells were previously thought to be precursor peritubular myoid cells, however, this has recently been shown not to be the case (Cool et al., 2008). Whilst the cords are forming, Leydig cells, also derived from the coelomic epithelium, differentiate and migrate into the spaces between the seminiferous cords (Sharpe, 2006). Testosterone produced by these fetal Leydig cells has a role in differentiation of the Wolffian duct into the epididymis, vas deferens and seminal vesicles (Sharpe, 2006). Anti-müllerian hormone, another important hormone, secreted by the developing Sertoli

cells, promotes regression of the Müllerian duct (in females, this would develop into the fallopian tubes and uterus) (Behringer et al., 1994).

The testes develop below the kidneys in the embryo. The testes must descend from the abdomen, through the inguinal canal into the scrotum as the fetus develops. This occurs in response to androgens and insulin-like growth factor 3, produced by the fetal Leydig cells (Sharpe, 2006). Testes finally complete descent into the scrotum at about postnatal day 20 in the rat (Vitale et al., 1973).

During postnatal and pubertal development, Sertoli cells change in morphology and function from the immature proliferative cells to mature non-proliferative cells (Sharpe et al., 2003). Differentiation and proliferation of the gonocytes also occurs, forming the spermatogonia that will undergo spermatogenesis. Fetal Leydig cells mature into adult Leydig cells accompanied by an increase in testosterone biosynthetic enzymes and a decrease in testosterone metabolism, resulting in production of five times more testosterone (Shan et al., 1996).

In the rat, the onset of spermatogenesis occurs at around postnatal day 8 (Vitale et al., 1973). Junction protein complexes form between adjacent Sertoli cells, creating the blood-testis barrier, dividing the tubule into two compartments, the basal, and adluminal compartments separating the pre- and post-meiotic germ cells. Formation of the functional blood-testis barrier occurs around postnatal days 15-19 in the rat (Vitale et al., 1973; Russell et al., 1989). This corresponds with the time that the first spermatocytes form.

2.3 The Adult Testis and Male Reproductive Tract

The function of the male reproductive system is to produce and release sperm to allow fertilisation of an ovum leading to the production of offspring. The adult male reproductive system consists of two testes, each joined to its own epididymis and connected to the penis via the vas deferens. Germ cells develop in the testes, and then

travel through the epididymis (caput to cauda) where maturation, including gain of motility, takes place. The epididymis connects to the vas deferens (Figure 2-1), which converges with the seminal vesicles at the site of the ejaculatory ducts. The sperm then travel through the urethra, ready for ejaculation. During sexual intercourse, the sperm are released, as semen, into the female reproductive tract, where the final stages of maturation occur (capacitation). This final stage of maturation leaves the sperm ready for fertilisation should an ovum be present. The sperm are protected during their journey from the epididymides into the female tract by seminal vesicle fluid and prostatic fluid. These three components make up semen.

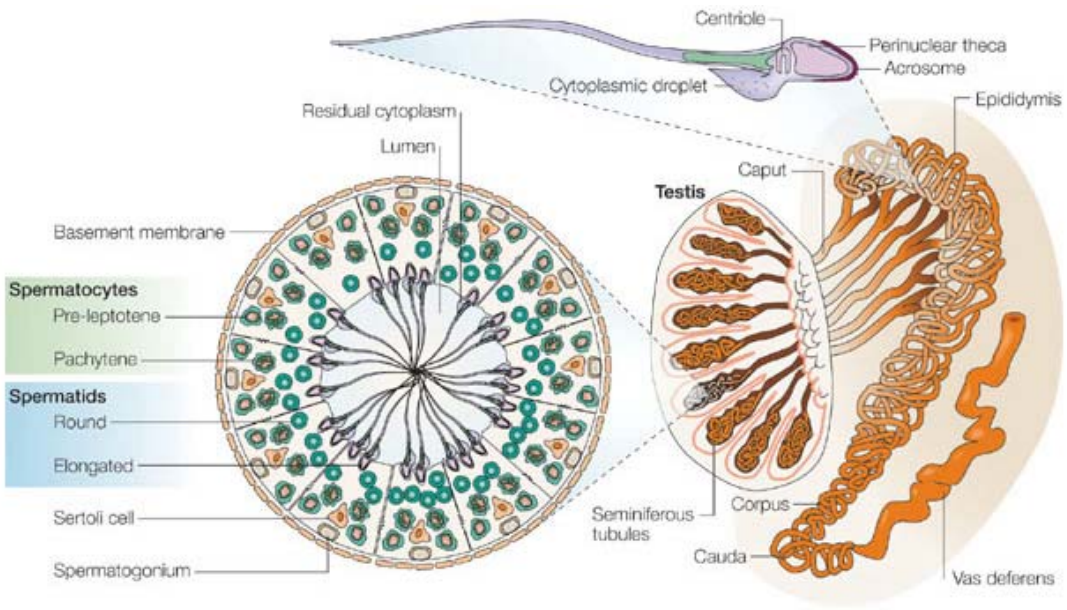


Figure 2-1 Structure of the testis, seminiferous tubule and spermatozoon. Spermatogenesis takes place in the seminiferous tubules located in the testes. Each end of the tubules drains into the Rete testis which connects to the epididymis. Here the spermatids released from the seminiferous tubules continue to develop into functionally mature spermatozoa. The cauda epididymis leads to the vas deferens. *from Krawetz, 2005.*

The main parts of the male reproductive tract are found outside of the body; with the testes and epididymis located in the scrotum. The scrotum functions to regulate the temperature of the testes between 2-7^{o}C cooler than the rest of the body, the temperature required for spermatogenesis (Setchell, 1970; Waites, 1970).

The focus of this review is testicular toxicity. Before adverse effects on the testis can be considered, an understanding of the structure and function of the testis is essential. This section of the review will focus on providing the background understanding of these areas.

2.3.1 The Testis

The testis has two functions; a reproductive function, the production of sperm, and an endocrine function, the production of testosterone. The testis can be generally split into two compartments, each of which focuses on one of these functions. The interstitial space contains the Leydig cells, responsible for testosterone production. The second compartment is the seminiferous tubules, comprised of lengths of tubules consisting of a seminiferous epithelium and lumen. It is here that spermatogenesis takes place. The tubules converge at the rete testis, a network of channels, which lies towards one pole of the testis in the rat, close to the surface. The efferent ducts connect the rete testis to the epididymis. In rats, the efferent ducts are long convoluted ducts, and are the primary site for fluid resorption. Both compartments of the testis are enclosed in a thick fibrous capsule, the tunica albuginea. The capsule has a contractile role to aid sperm transport and a role in maintenance of pressure inside the testis (Setchell et al., 1994).

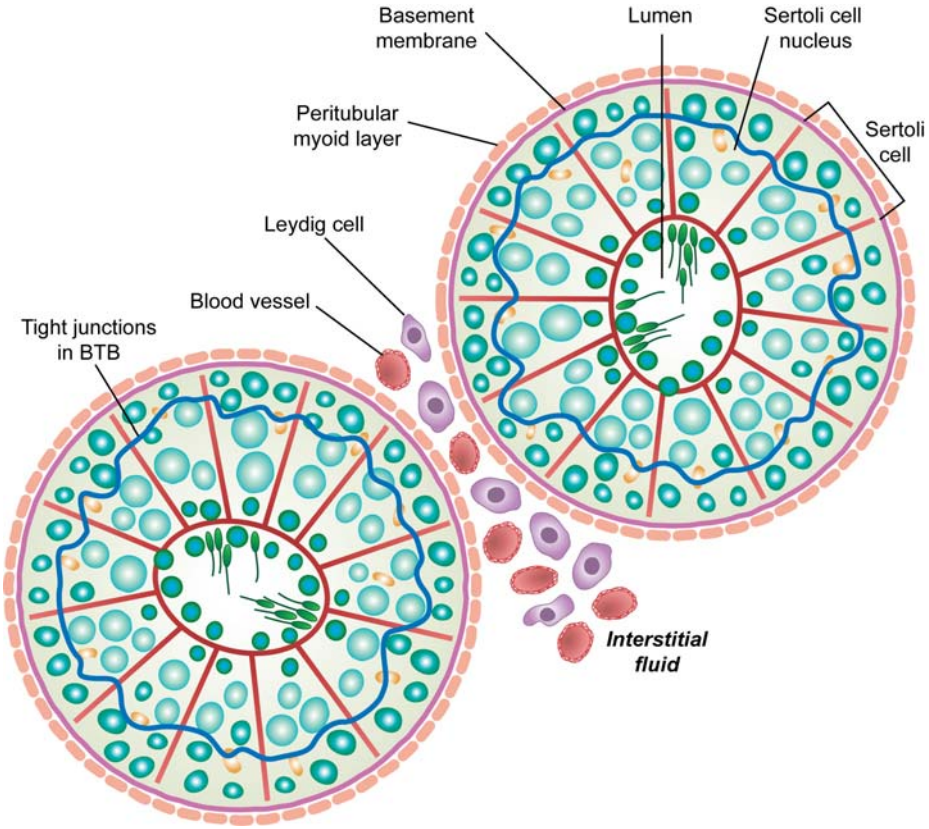


Figure 2-2 Diagrammatic representation of cross-section of seminiferous tubule structure showing the site of the blood-testis barrier, and the Leydig cells and blood vessels in the interstitial space.

2.3.2 Interstitial Space

The testicular interstitial space is the site of testicular endocrine function. The space between the seminiferous tubules, contains loose connective tissue with Leydig cells, blood vessels, lymphatics (Fawcett et al., 1973) and leukocytes (Hedger, 1997) (Figure 2-2).

2.3.2.1 Leydig Cells

The Leydig cells make up about 10-60% of the tissue in the interstitial space, depending on species (Setchell et al., 1994). Leydig cells are typically irregular in shape, and clustered around blood vessels (Clark, 1976). Steroid hormone synthesis takes place in the Leydig cells, and the dominant organelle in these cells is smooth

endoplasmic reticulum which has a vast surface area with binding sites for enzymes for steriodogenic conversions (de Kretser and Kerr, 1994). The most important hormone produced by the Leydig cells is testosterone, although oestrogens, insulin like factor 3 (insl3), cytokines and oxytocin are also produced (Sharpe et al., 1990; Hall, 1994; Ivell and Bathgate, 2002).

Testosterone has an important role in the testis as it controls spermatogenesis, as well as regulating and maintaining masculinisation and development. Testosterone production is regulated by the secretion of pulses of LH from the pituitary (Hall, 1994). LH has both acute and long-term effects on the Leydig cells and testosterone production (Zirkin et al., 1997). Long-term effects of LH include the maintenance of steroidogenic enzymes, and Leydig cell volume and structure, whereas acute effects involve activation of signalling cascades to initiate testosterone production (Zirkin et al., 1997).

Testosterone is synthesised from cholesterol, which is either synthesised in the endoplasmic reticulum or mobilised from the plasma membrane or lipid droplets (Rone et al., 2009). Activation of adenylate cyclase following binding of LH to its receptor in the Leydig cell plasma membrane causes an increase in intracellular cAMP. This leads to an increase in lipid synthesis, protein synthesis and protein phosphorylation, all of which assist with the translocation of cholesterol to the mitochondria (Rone et al., 2009). Delivery of cholesterol to the inner mitochondrial membrane is the rate-limiting step in steriodogenesis (Crivello and Jefcoate, 1980). Therefore regulation of testosterone synthesis must be controlled by factors involved in translocation of cholesterol. Cholesterol is thought to be targeted to the mitochondria via a combination of cholesterol-binding proteins and endosomes/lysomes (Rone et al., 2009). Many proteins have been studied in association with cholesterol translocation in steroidogenesis. Rone et al., 2009, reviewed current ideas regarding protein-protein interactions in cholesterol translocation. A range of proteins have been implicated including START domain proteins such as MLN64 and steroidogenic acute regulatory protein (StAR) with

roles in targeting cholesterol to the mitochondria, and translocator protein (TSPO) (previously referred to as peripheral benzodiazepine receptor (PBR)), with a role in importing cholesterol to the inner mitochondrial membrane. The mechanism proposed involves the binding of cholesterol to StAR located in the outer mitochondrial membrane, which promotes a conformational change in the protein and in the outer mitochondrial membrane. This interaction could promote clustering of TSPO and its associated proteins (including VDAC, DBI and PAP7), which connect the outer and inner mitochondrial membranes allowing transfer of cholesterol to the inner membrane (Rone et al., 2009).

At the inner mitochondrial membrane, cholesterol associates with the P450scc (or CYP11A1) enzyme, and is converted to pregnenolone via a series of hydroxylation reactions (Hall, 1994). Pregnenolone is translocated from the mitochondria to the smooth endoplasmic reticulum (SER), where it binds to 3β-hydroxysteroid dehydrogenase (3β-HSD) and is converted to progesterone. Progesterone is then converted to 17α-hydroxyprogesterone, and then to androstenedione, catalysed by 17α-hydroylase and C17-20 lyase, respectively. 17β-hydroxysteroid dehydrogenase promotes the conversion of androstenedione to testosterone, also in the SER (Hall, 1994; Zirkin et al., 1997).

Testosterone synthesised in the Leydig cells is secreted and transported in the blood and lymph or to the seminiferous tubules via the interstitial fluid. In the seminiferous tubules, testosterone can pass freely through the epithelium into the lumen, where it binds with androgen binding protein and is carried into the efferent ducts (Johnson, 2007). Testosterone can also be converted to oestrogen (Hall, 1994). Bergh, 1982, suggested that Leydig cells vary in size throughout a Leydig cell cycle, thought to be regulated by factors secreted by Sertoli cells in a stage specific manner, however, Fouquet, 1987, observed no significant changes in Leydig cell size across the spermatogenic cycle but noted an increase in SER volume density at stages VII-VIII (compared to stages XI-XII) in adult rats.

In humans, two populations of Leydig cells exist, fetal Leydig cells, responsible for masculinisation and testicular development during fetal life, and adult Leydig cells, which become apparent at puberty. The number of fetal Leydig cells rapidly declines after birth, while the adult population expands at puberty (reviewed in Griswold and Behringer, 2009).

2.3.2.2 Blood Vessels

Supply of blood is essential to all organs, to transport oxygen and nutrients to the cells, and transport waste products away from the cells. In the testes, a rich hexagonal and pentagonal columnar network of capillaries surrounds the seminiferous tubules (Murakami et al., 1989). This capillary bed is derived from the testicular artery and drains via subalbugineal and intra-albugneal veins into the pampiniform plexus and into the testicular vein (Murakami et al., 1989).

Two types of capillaries have been described in the rat testis. Intertubular capillaries or 'Zwickelcappillaren' are found in the triangular interstitial spaces between three adjacent seminiferous tubules (Kormano, 1967). These vessels are encircled by (Clark, 1976), and supply (Murakami et al., 1989) the Leydig cells. Larger blood vessels, the peritubular capillaries or 'Quercapillaren' run parallel along the long axes of the seminiferous tubules near the basement membranes, in a rope like structure (Kormano, 1967). These peritubular capillaries supply the seminiferous tubules (Murakami et al., 1989). Intertubular venules drain both types of capillaries (Kormano, 1967). In humans, the structure of the testicular vasculature is less organised, with the capillaries forming an irregular plexus around the tubules (Suzuki and Nagano, 1986).

2.3.2.3 Lymphatics

The testicular lymphatic system varies depending on species. Fawcett et al., 1973, compared the interstitial space in 14 mammalian species, and found that in rats, there

is a relatively small volume of Leydig cells and connective tissue, with extensive lymphatic sinusoids with discontinuous endothelial lining. This means that a large part of the interstitial cells are directly exposed to the lymph. In contrast, in humans, the lymphatic vessels are centrally located in the interstitial spaces with clusters of Leydig cells (Fawcett et al., 1973). The three-dimensional organisation of the lymphatics in the rat testis was considered by Clark, 1976, who reported that endothelial lined lymphatic sinusoids are located between lengths of adjacent seminiferous tubules (along the long axes), whereas open sinusoids are found in the triangular interstices between three adjacent tubules. A lymphatic space was noted around each tubule with free communication via fenestrae to the interstitial fluid (Clark, 1976). Clark, 1976, also reported that substances from Leydig cells and blood vessels must, therefore, pass through the interstitial fluid into the lymphatic fluid and then into the seminiferous tubules. The lymphatic sinusoids drain into lymphatic collecting vessels and emerge from the capsule at three points. These three lymphatic vessels join to form a single collecting trunk (Perezclavier and Harrison, 1978).

2.3.2.4 Leukocytes

Testicular leukocytes consist mainly of macrophages, although lymphocytes and mast cells are also present (Hedger, 1997). Macrophages make up about 25% of the cells in the interstitum in the rat (Niemi et al., 1986). The exact role of macrophages and other leukocytes in the testis is yet to be completely understood. Macrophages are located in close proximity to Leydig cells, and the complex cytoplasmic processes of Leydig cells have been shown to extend to adjacent macrophages (Miller et al., 1983; Hutson, 1992). The macrophages are thought to have a role in Leydig cell function with a positive effect on steriodogenesis (reviewed in Hedger, 1997) and a potential role in Leydig cell development (Raburn et al., 1993). A second role for the interstitial macrophages is that they have an immunoregulatory role involved with maintenance of immune-privilege in the testis (reviewed in Hedger, 2002). Finally a third role for resident macrophages in the interstitial space is to increase capillary permeability (Setchell et al., 1994).

2.3.2.5 Interstitial Fluid

Testicular interstitial fluid is derived from the blood plasma, and in the rat, is continuous with the lymphatic system (Fawcett et al., 1973). The interstitial fluid has an important role for transport and communication in the testis, and is similar in composition to plasma. The seminiferous tubules are avascular so transport of nutrients and hormones from the blood vessels and interstitial cells must take place via the interstitial fluid. Formation of interstitial fluid is balanced by removal of the fluid to the bloodstream via either resorption into venules or by lymphatic drainage (Setchell et al., 1994). Interstitial fluid is also taken up by the Sertoli cells and resecreted with a different composition as seminiferous tubule fluid, to transport factors from the Sertoli cells to the germ cells (Sharpe et al., 1996). The control of interstitial fluid production is, therefore, important. It is thought to be regulated by testosterone, as a consequence of its effects on vasomotion and blood flow. It is also thought that elongate spermatids may have a role in regulation of testicular fluid dynamics, likely to be via their effect on the Sertoli cells (Sharpe et al., 1996).

2.3.3 Seminiferous Tubules

The seminiferous tubules are the site for the second testicular function; spermatogenesis. Seminiferous tubules are long lengths of cylindrical tubules, consisting of a seminiferous epithelium made up of somatic Sertoli cells, and developing germ cells. A central lumen contains seminiferous tubule fluid. A basement membrane and layer of peritubular myoid cells surround and enclose the seminiferous epithelium (Figure 2-2). These function as a partial barrier between the interstitial space and seminiferous epithelium, and the peritubular myoid cells have been shown to have a contractile function, thought to promote movement of the seminiferous tubule fluid through the tubules or to have a role in movement and release of spermatids from the epithelium (Suvanto and Kormano, 1970). A second, and fundamentally more important barrier in the seminiferous epithelium exists between adjacent Sertoli cells and is termed the blood-testis barrier (BTB) (Dym and Fawcett, 1970). This splits the seminiferous tubules into two compartments, the

basal compartment and the adluminal compartment. The site of the blood-testis barrier is shown in Figure 2-2.

In rats, the seminiferous tubules are straight and bundled parallel to each other except in turning areas where they bend sharply in hairpins (Clermont and Huckins, 1961; Suzuki and Nagano, 1986), whereas in humans, seminiferous tubules are more convoluted, although they become straighter towards the collecting ducts, and are arranged in lobules (Gier and Marion, 1970).

2.3.4 Spermatogenesis

Spermatogenesis is the process by which spermatozoa are formed from germ cells. It is a complex process involving different stages of differentiation as the cells move towards the lumen of the seminiferous tubules for release and collection in the later sections of the reproductive tract. Spermatogenesis can be divided into three phases (Sharpe, 1994):

- Proliferative Phase – production of a large numbers of cells via a series of mitotic divisions
- Meiotic Phase – generation of haploid cells
- Spermiogenic Phase – differentiation of haploid cells into the complex and compact structure of mature spermatozoa and release from the seminiferous epithelium (spermiation)

2.3.4.1 Proliferative Phase

The proliferative phase (or spermatogonial phase) involves the initial division of spermatogonial stem cells (termed A_0 in the rat and A_d in humans) into two daughter cells, one of which continues the process of spermatogenesis and the other remains as a stem cell (Sharpe, 1994). In rats, A_0 spermatogonia undergo a series of sequential mitotic divisions producing four generations of A spermatogonia (termed

A_1-A_4), one generation of intermediate type spermatogonia, B spermatogonia, and finally, preleptotene primary spermatocytes (Figure 2-3).

The number of mitotic divisions is characteristic for each species. In humans, A_d (dark) spermatogonia undergo two mitotic divisions, forming A_p (pale) spermatogonia and then type B spermatogonia, before giving rise to primary spermatocytes (Clermont, 1972).

The proliferative phase of spermatogenesis takes places in the basal compartment of the seminiferous tubules. Primary spermatocytes (preleptotene-leptotene) cross the blood-testis barrier, and all meiotic and post meiotic germ cells reside in the adluminal compartment (Sharpe, 1994).

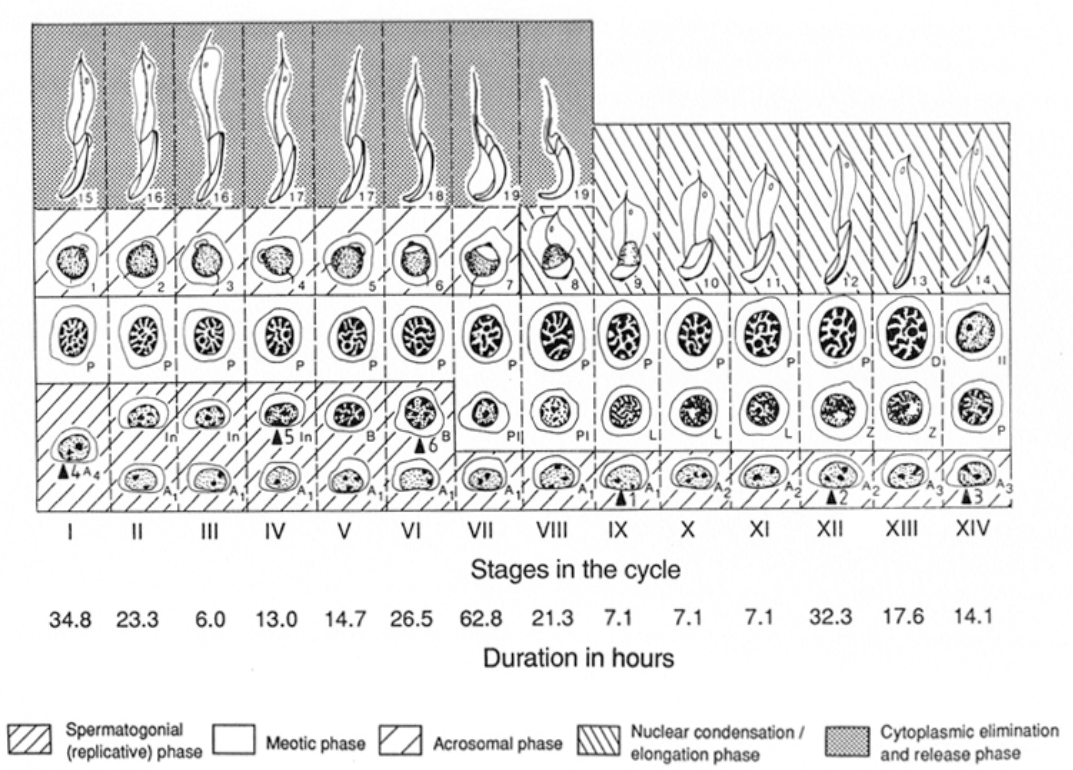


Figure 2-3 The Rat Spermatogenic Cycle. The spermatogenic cycle is divided into 14 stages in the rat, described by the complement of germ cells associated with the Sertoli cells at each stage. Each vertical column represents each different stage and the germ cells present in the seminiferous epithelium, with the lumen of the tubule to the top, and the basement membrane to the bottom of the diagram. Each stage lasts for a fixed period of time, shown underneath each column. Spermatogenesis is divided into three phases; the spermatogonial phase (A-spermatogonia (A_1-A_4), Intermediate spermatogonia (In), B-spermatogonia (B)), the meiotic phase (preleptotene (Pl), leptotene (L), zygotene (Z), pachytene (P), diplotene (D) primary spermatocytes, and secondary spermatocytes (II)), and the spermiogenic phase divided into the acrosomal phase, nuclear condensation/elongation phase, and cytoplasmic elimination and release phase (round spermatids (1-7), elongate spermatids (8-19)). *from Sharpe, 1994* .

2.3.4.2 Meiotic Phase

Meiosis is the process of cell division by which haploid gametes are formed from diploid cells, and is often known as a reduction division. The prophase I phase of meiosis is prolonged, and produces a sequence of types of spermatocyte which can be identified by the nature of their nuclei. Homologous recombination takes place during prophase I, and is an important source of genetic variation. During the leptotene stage of prophase I, condensation of chromosomes occurs. Synapsis of

homologous chromosomes takes place during the zygotene stage. The pachytene stage is the longest stage of prophase; therefore pachytene spermatocytes are present in seminiferous tubules at most spermatogenic stages (Figure 2-3, and section 2.3.4.4). Chromosomal crossover occurs during the pachytene stage. During the diplotene stage, degradation of the synaptonemal complex occurs, followed by a slight separation and condensation of homologous chromosomes, and some DNA transcription (Clermont, 1972). The first meiotic division completes through metaphase I (alignment of chromosomes), anaphase I (separation of homologous chromosomes), and telophase I (formation of daughter cells) to form two secondary spermatocytes. These cells are short-lived and rapidly enter the second meiotic division to form haploid round spermatids (Sharpe, 1994).

2.3.4.3 Spermiogenic Phase

The spermiogenic phase of spermatogenesis involves the differentiation of each round spermatid into a spermatozoon and its release from the seminiferous epithelium. This process involves remarkable remodelling of the cells.

Spermiogenesis is usually divided up into stages, although the different stages do overlap each other (Sharpe, 1994). Round spermatids must change shape to form elongate spermatids. The flagellum elongates forming a tail, which is connected to the mid-piece, where the cells' mitochondria accumulate. As the spermatids differentiate, the acrosome develops at the tip of the sperm head (Figure 2-1). This is an important part of the mature spermatozoon, as it contains lysosomal enzymes which are released on attachment to an ovum and allow breakdown of the zona pellucida to permit fertilisation.

In the spermatid nucleus, condensation of the haploid chromosomes occurs. Histones are replaced by transition proteins, which are then replaced by protamines (Wouters-Tyrou et al., 1998), creating compact chromatin fibres that can be packaged in parallel arrays in the spermatid head. Condensation of the chromosomes results in

transcriptionally inactive DNA. Therefore mRNA for all the proteins required during spermiogenesis and later stages of maturation leading to fertilisation must be transcribed before nuclear condensation.

There is a reduction in the amount of cytoplasm in the spermatid, which is removed in the form of the residual body. This is later phagocytosed by the Sertoli cells, and drawn down through the epithelium to the base of the Sertoli cell where it is degraded lysosomally (Sharpe, 1994). Release of excess cytoplasm in the form of the residual body, marks the final stage of spermatid differentiation. Spermatids are released from the apex of the Sertoli cells into the tubular lumen, in a process known as spermiation. The released spermatozoa are washed along the seminiferous tubules in fluid secreted by the Sertoli cells (Johnson, 2007). Any spermatids that are not released are phagocytosed by the Sertoli cells (Sharpe, 1994).

2.3.4.4 Organisation of Spermatogenesis

So far, this description of spermatogenesis has not explored one of the most important aspects of spermatogenesis; the organisation of the germ cells at the different stages of development in the seminiferous epithelium.

The duration of the spermatogenic cycle, or length of time between each wave of spermatogenesis, is constant and species specific. In the rat, the spermatogenic cycle is 12.5-13.3 days compared to 16 days in man (Sharpe, 1994). This is a much shorter time period than the duration of spermatogenesis itself (time for one A_0 spermatogonium to differentiate into a spermatozoon); 51.6-53.2 days in the rat and 74-76 days in humans (Sharpe, 1994). The length of the spermatogenic cycle is clearly shorter than the duration of spermatogenesis, so successive spermatogenic processes must be going on at the same time.

Throughout spermatogenesis, cells are progressively displaced, moving towards the lumen of the seminiferous tubules, as less advanced cells reach a new stage of

development. In a cross section of a seminiferous tubule it is, therefore, possible to see several germ cells at different stages of development, layered concentrically around the tubule. As both the spermatogenic cycle and duration of spermatogenesis are fixed, cells in successive cycles will always develop in parallel (Johnson, 2007). The spermatogenic cycle is divided up into stages dependent on the types of germ cells present in the seminiferous tubules at each stage. There are 14 stages in the rat, and 6 in humans (Figure 2-3).

The second consideration for organisation of spermatogenesis in the seminiferous tubules is the arrangement of these spermatogenic stages along the lengths of the tubules. Spermatogenesis commences at different times in different areas of the tubules, resulting in a continuous release of spermatozoa rather than a coordinated release that would be present if all the spermatogonia commenced spermatogenesis at the same time. In the rat, the spermatogenic stages are arranged in segments along the length of the tubules, with consecutive stages usually adjacent to each other. In the human, marmoset, and a few other primates (but not all), seminiferous tubules have a very different organisation, with a mixed arrangement of spermatogenic stages, where in a cross section of a tubule, 'wedges' of different stages can be observed (Schulze and Rehder, 1984).

A further consideration in the organisation of spermatogenesis in the seminiferous tubules is the duration of each stage. This means that along the length of a seminiferous tubule, sections of the tubule at each stage will be proportional to the duration of the stage. Therefore, in a testis section, you are more likely to see tubules of long duration stages than of short duration stages, because longer portions of tubules will be present for the former than the latter.

2.3.4.5 Regulation of Spermatogenesis

Spermatogenesis is regulated by a combination of endocrine, paracrine and autocrine pathways. Follicle Stimulating Hormone (FSH), Luteinising Hormone (LH), and

testosterone all have a role in the control of spermatogenesis. The gonadotrophins, LH and FSH are secreted by the pituitary in response to hypothalamic gonadotrophin-releasing hormone (GnRH) (McLachlan et al., 2002). FSH binds to Sertoli cells in a stage-specific manner. It is thought to have effects on the Sertoli cell cytoskeleton, which in turn affects the ability of germ cells to bind to the Sertoli cells (McLachlan et al., 1995). FSH appears to support germ cell development up to the round spermatid stage (McLachlan et al., 1995). FSH also has a role in Sertoli cell proliferation during testicular development (Holdcraft and Braun, 2004). FSH secretion is regulated by negative feedback via the testicular hormone, inhibin B (Hayes et al., 2001b) and via testosterone, following aromatisation to estradiol (Hayes et al., 2001a).

LH acts on spermatogenesis by promoting secretion of testosterone from Leydig cells (Sharpe, 1994). Testosterone acts by binding to androgen receptors (ARs), promoting nuclear translocation and subsequent transcriptional regulatory affects. ARs are expressed in Leydig cells, peritubular myoid cells, and in the Sertoli cells, and in the latter this expression is stage-dependent being greatest at stage VII in the rat. Recently, the role of AR in the Sertoli cells has been investigated using Sertoli cell AR knockout mice. The results showed an essential role for AR-mediated affects on Sertoli cells for the progression of spermatocytes through meiosis I, the transition of round spermatids to elongate spermatids, and during the terminal stages of spermiogenesis (reviewed in Holdcraft and Braun, 2004). A similar approach has also been used to elucidate the role of AR in the peritubular myoid cells. Specific knockout of AR in peritubular myoid cells in mice resulted in disrupted sperm production, with a decrease in all types of germ cells, suggesting that androgen action on the peritubular myoid cells is also essential for spermatogenesis (Welsh et al., 2009).

2.3.5 The Sertoli Cell

Sertoli cells are large irregular columnar epithelial cells in the seminiferous epithelium. The cells are polarised and each extends from the basement membrane to the lumen of the seminiferous tubule (Figure 2-1, Figure 2-2). Sertoli cells surround the developing germ cells, and interact with the germ cells via a range of junction protein complexes (Figure 2-4). Sertoli cells also form junction complexes between adjacent cells, forming the blood-testis barrier, which divides the tubules into the basal and adluminal compartments (Figure 2-4). The Sertoli cells must undergo many changes in conformation as the developing germ cells displace more advanced cells at each stage of spermatogenesis. The Sertoli cell cytoskeleton therefore has an important function. The cytoskeleton is made up of actin filaments, microtubules and intermediate filaments, all of which affect the cell shape, and location of intracellular organelles and intercellular junctions. A periodic cyclic reorganisation of the Sertoli cell cytoskeleton takes place in association with germ cell maturation (reviewed in Boekelheide et al., 1989). The cytoskeletal components form complex, highly organised structures to physically support the germ cells, and promote germ cell movement (Boekelheide et al., 1989).

Each Sertoli cell can only support a fixed number of germ cells so the number of Sertoli cells is directly related to sperm counts (Monsees et al., 2000). About 1:50 (Sertoli:germ cells) are found in adult male rats (Mruk and Cheng, 2004). Germ cells must remain attached to Sertoli cells throughout all stages of spermatogenesis (Mruk and Cheng, 2004). Extensive interactions are present between the Sertoli cells and germ cells including adherens junctions, ectoplasmic specialisations, gap junctions and desmosome like junctions (Figure 2-4). Coordination of these interactions with the Sertoli cell cytoskeleton is essential for movement of germ cells across the seminiferous epithelium during spermatogenesis

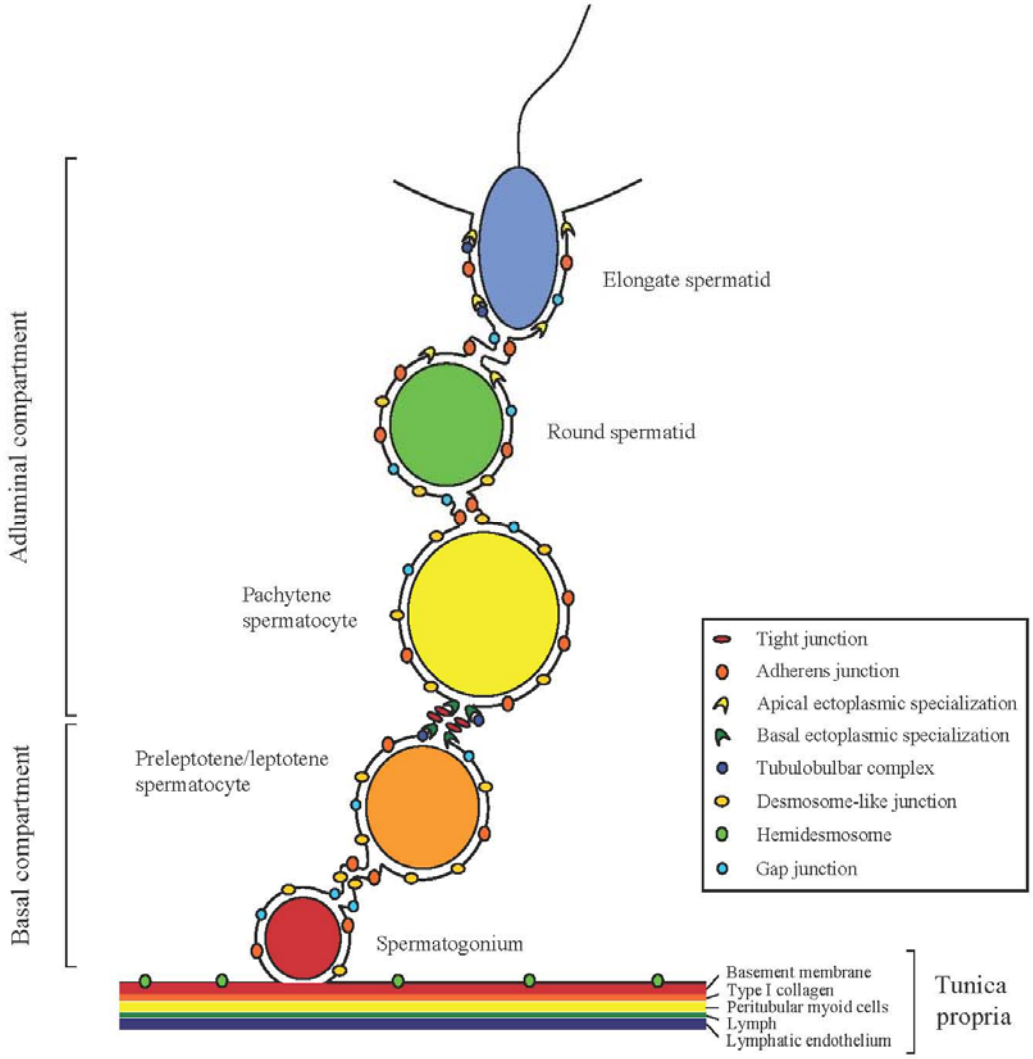


Figure 2-4 Diagrammatic representation of the different stages of germ cells and their relative location in the seminiferous tubule, and the junction complexes formed between Sertoli-germ cells and Sertoli-Sertoli cells. *Reproduced with permission of C.Yan Cheng, originally from (Mruk and Cheng, 2004).*

The Sertoli cells have an important role in nurturing the developing germ cells. They must maintain the spermatogonia, and may have a role in control of spermatogenesis and spermiation. They also have a role in phagocytosis of residual bodies. Due to the divided nature of the seminiferous tubule (basal and adluminal compartments), with the formation of the blood-testis barrier between adjacent cells, the Sertoli cells must pass nutrients and oxygen from the interstitial space to the developing germ cells, as

well as providing hormonal support for the germ cells. The importance of the blood-testis barrier will be discussed in more detail in the next section.

Current thinking regarding the relationship between Sertoli cells and germ cells suggests that although Sertoli cells are important for germ cell nurture and development; this is achieved by providing an appropriate environment in which the germ cells can undergo their endogenous programmed development. However, paracrine signalling must take place between the germ cells and Sertoli cells to allow coordination of changes to the (Sertoli-cell regulated) environment and this is thought to be controlled by the germ cells signalling to Sertoli cells to regulate their function (Sharpe, 1993).

2.3.6 The Blood-Testis Barrier

Although a barrier to the penetration of substances entering the seminiferous tubules had been identified previously, it was Dym and Fawcett, 1970, who reported the location of the barrier to be between adjacent Sertoli cells. It is formed by junctional complexes, on the luminal side of the preleptotene spermatocytes (Figure 2-2), separating the seminiferous tubule into the basal and adluminal compartments. This division in the seminiferous tubule creates a specialised environment for post-meiotic development of germ cells, as well as regulating the passage of molecules to and from the aduminal compartment (Mruk and Cheng, 2004). The blood-testis barrier has a further role in acting as an immunological barrier (Mruk and Cheng, 2004), ensuring that the immune system does not recognise and produce antibodies to the antigens on the cell surfaces of the haploid germ cells. During testicular development, the formation of the BTB is required for lumen formation and onset of fluid secretion (Vitale et al., 1973).

The blood-testis barrier is unusual in its composition in comparison to the blood-brain, blood-retina and blood-epididymal barriers which have tight junctions at the apical portion, followed by adherens junctions. The blood-testis barrier consists of

co-existing tight junctions, adherens junctions and desmosome like junctions (Yan and Cheng, 2005). The blood-testis barrier also has an important role in seasonal breeders, with cyclic degeneration and reformation of the barrier following the spermatogenic phases (Pelletier, 1986).

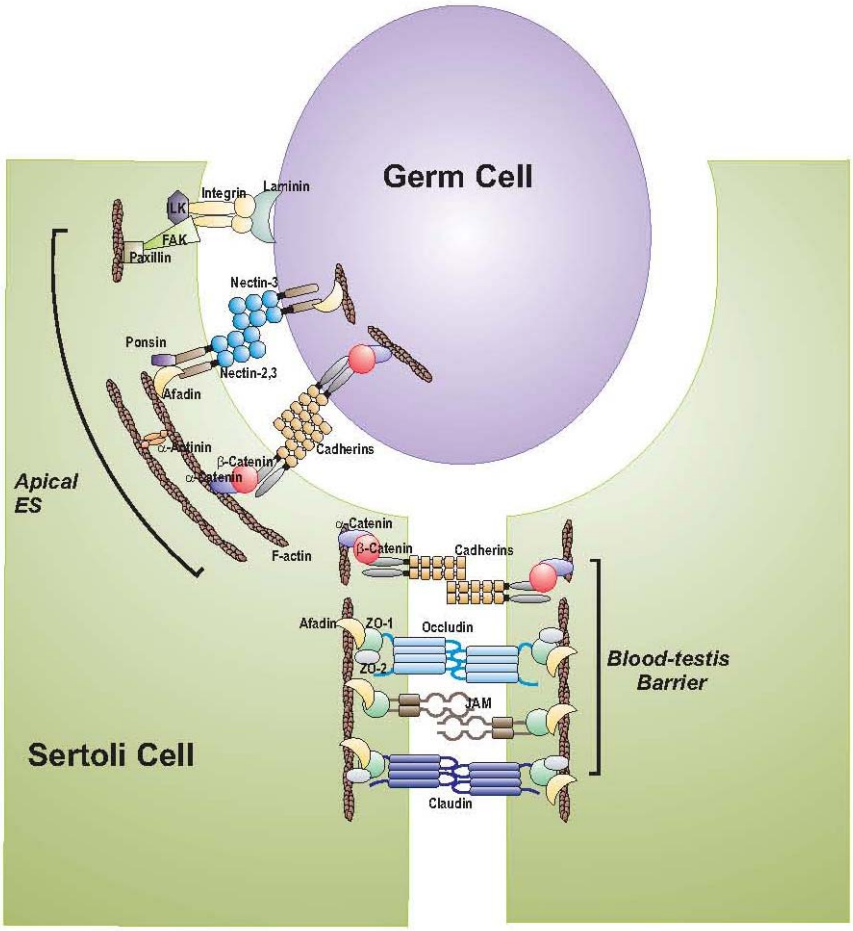


Figure 2-5 Detailed diagram of junction complexes between Sertoli cells and germ cells (apical ectoplasmic specialisations (ES)) and adjacent Sertoli cells at the site of the blood-testis barrier. *Reproduced with permission of C.Yan Cheng, originally from Xia et al., 2005.*

2.3.6.1 Tight Junctions

Tight junctions are an example of occluding junctions. In the testis, they are areas of close contact between plasma membranes of neighbouring cells, composed of fibrils

that encircle the basal domain of each cell. These fibrils represent a row of tightly arranged transmembrane proteins that are sensitive to rapid changes in the lipophilic nature of the plasma membrane (reviewed in Mruk and Cheng, 2004).

There are three groups of integral membrane proteins making up the testicular tight junction; occludin, claudins, and junction adhesion molecules (JAMs) (Figure 2-5). A further recently identified tight junction protein is coxsackievirus and adenovirus receptor (CAR), although its role in the blood-testis barrier has yet to be elucidated (Mruk and Cheng, 2004). These connect to adaptor proteins, such as zonula occludens family members, which in turn link to the Sertoli cell cytoskeleton.

2.3.6.1.1 Occludin

The best-studied tight junction protein is occludin (Mruk and Cheng, 2004). It was first described by Furuse et al., 1993. Occludin is a 65kDa transmembrane protein with two extracellular loops and one intracellular loop, and four transmembrane domains, with both N-terminal and C-terminal ends situated in the Sertoli cell cytoplasm (reviewed in Lui et al., 2003).

The first extracellular loop of the protein is involved in cell-cell adhesion, while the second loop appears to be required for the formation of the tight junction barrier. Chung et al., 2001, showed that a synthetic peptide corresponding to 22 amino acids in the second loop administered to adult male rats by intra-testicular injection led to reversible infertility due to interference of the peptide with occludin-occludin interactions in the blood-testis barrier resulting in failure of the barrier and germ cell loss. Deletion of the occludin gene in mice (Saitou et al., 2000) produced histological abnormalities in the testes only in aged mice (40 to 60 weeks compared to 6 weeks), with seminiferous tubule atrophy and depletion and loss of germ cells observed. However, sexual behaviour appeared to be affected with occludin$^{-/-}$ males, with apparent normal spermatogenesis, failing to produce litters with wild-type females

(Saitou et al., 2000). Occludin, therefore, appears to have an important role in testis function.

2.3.6.1.2 Claudins

The claudin proteins have a similar structure to occludin, with a smaller second extracellular loop, and smaller cytoplasmic domain. They are also smaller in size (20-24kDa). The superfamily of claudins consists of at least 24 members (Mruk and Cheng, 2004), expressed in different organs, with different C termini suggesting that their organ specificity might be due to their association with different cytoplasmic proteins.

Seven of the 24 claudins have been identified in the testis, claudins 1, 3, 4, 5, 7, 8 and 11 (reviewed in Mruk and Cheng, 2004). However, claudin 11 is the best studied. Gow et al., 1999, showed that ablation of claudin-11 in mice produced sterile claudin-11 null males with smaller testes than wild-type. Histological analysis of the testes showed narrowing of the seminiferous tubules, an absence of spermatozoa, and ill-defined lumens filled with aggregates of Sertoli cells. Other tight junction proteins (occludin, ZO-1) were shown to be present at the site of the blood-testis barrier in claudin-11 null mice suggesting that knockout of claudin-11 does not affect recruitment of proteins to the BTB, but has an effect on establishment of the barrier and therefore on spermatocyte maturation, as well as a role for claudin-11 in Sertoli cell contact with the basement membrane and long term survival of the Sertoli cells (Gow et al., 1999). Their results suggested that claudin-11 is essential for forming the parallel organisation of the tight junction strands in a subset of tight junctions found in the testis and in the central-nervous system. This finding highlights that there are likely to be specific functions for the different members of the claudin family. Mazaud-Guittot et al., 2010, further investigated the testicular phenotype in claudin-$11^{-/-}$ mice. Their results showed that the Sertoli cells in claudin-11 null mice form clusters and detach from the basement membrane. The detached Sertoli cells acquire a fibroblastic shape in the lumen of the tubules, which is likely

to be due to altered cytoplasmic gene expression. The altered Sertoli cell aggregates were shown to be cleared through the seminiferous tubule lumen and detected in the epididymis. These results suggest a role for claudin-11 in maintenance of Sertoli cell epithelial differentiation (Mazaud-Guittot et al., 2010).

2.3.6.1.3 Junction Adhesion Molecules

Junction adhesion molecules (JAMs) are a member of the Ig superfamily and are one of the most recently described components of tight junctions (Fanning et al., 1999). Three JAMs have been identified, JAM-A, B and C (also termed JAM-1, 2, and 3) with roles in the testis, and a further two (JAM-4 and JAM-L) with roles in other tissues (Mandell and Parkos, 2005). JAM proteins consist of an N-terminal signal peptide, an extracellular domain with two Ig like domains, a single membrane spanning domain, and a short cytoplasmic tail (Mandell and Parkos, 2005). JAMs have been implicated in cell adhesion and cellular polarity, via homophilic and heterophilic interactions (with JAM family members or with other adhesion molecules) (Mandell and Parkos, 2005).

JAMs are thought to have a role in the regulation of tight junctions. Bazzoni et al., 2000, showed that JAM associates with the tight junction proteins ZO-1, cingulin, and occludin, and may bind and shuttle occludin to the membrane where they can interact with junction complexes, or alternatively, may be involved in ZO-1 mediated recruitment of occludin to the membrane. Deletion of *JAM-A, B*, and *C* genes has been investigated in mouse models. As well as a role in the BTB, JAM-A has been shown to have a role in sperm tail formation and is essential for normal sperm motility (Shao et al., 2008). However, disruption of the *JAM-A* gene caused no effect on testis size, the number of sperm produced or sperm morphology (Shao et al., 2008). Similarly, deletion of *JAM-B* (found at the site of the BTB), showed no impairment to the function of spermatozoa, with no effects observed on spermatogenesis or testis size (Sakaguchi et al., 2006). Gliki et al., 2004, showed that JAM-C has a role in Sertoli cell-spermatid interactions and is not present at the BTB.

Round spermatids in *JAM-C*$^{-/-}$ mice failed to polarise, suggesting a role for JAM-C in the differentiation of round spermatids into spermatozoa, which was shown to be due to its role in recruitment of a cell polarity complex to the spermatids (Gliki et al., 2004). JAMs are also known to have a direct role in the regulation of immune cell transport, by modulating the migration of neutrophils and monocytes in the epithelium (Fanning et al., 1999; Lui et al., 2003). The roles of the JAM family members appear to be varied, and for some protein members, essential for spermatogenesis.

2.3.6.1.4 Adaptor Proteins

Adaptor proteins interact with the intracellular side of the transmembrane tight junction proteins and link them to the Sertoli cell cytoskeleton. Zonula occludens 1 (ZO-1), ZO-2 and ZO-3 are examples of adaptor proteins. They are members of the membrane-associated guanylate kinase (MAGUK) protein family (Lapierre, 2000). ZO-1 is thought to have both signalling and scaffolding functions, and to directly interact with the C terminus of occludin and actin (Fanning et al., 1998). ZO-1 has also been shown to interact with α-catenin, showing a role in adherens junctions as well as tight junctions (reviewed in Mruk and Cheng, 2004).

In the testis, ZO-1 has been localised to the site of the blood-testis barrier, and is also reported to be located at Sertoli cell ectoplasmic specialisations adjacent to early elongating spermatids (Byers et al., 1991). There are two isoforms of ZO-1, ZO-1α^- and ZO-1α^+. They have been shown to associate differently with tight junctions, with ZO-1α^- expressed in the testis during adulthood (Balda and Anderson, 1993) and connecting to F-actin, and ZO-1α^+ expressed during puberty, linking to G-actin (Pelletier et al., 1997). ZO-1 interacts with a range of proteins, including ZO-2, ZO-3 (although this has not been identified in the testis so far), transcription factors, gap junction proteins, signalling molecules such as Rab, claudins, actin and cingulin, another adaptor protein (reviewed in Mruk and Cheng, 2004).

Other tight junction associated proteins include cingulin (Citi et al., 1988), which links to myosin, symplekin (Keon et al., 1996), and 7H6 (Zhong et al., 1993).

2.3.6.2 Adherens Junctions

Adherens junctions are a type of anchoring junction (alongside focal adherens, desmosomes, and hemidesmososmes). Adherens junctions form bridges between adjacent Sertoli cells, and Sertoli-germ cells, by binding the actin cytoskeleton of one cell to the other (Figure 2-4, Figure 2-5). They may also have a role in signal transduction. Three different types of adherens junctions exist in the testes; cadherin-catenin complexes, nectin-afdin-ponsin complexes, and integrin-laminin complexes (reviewed in Mruk and Cheng, 2004).

The cadherins are the best studied adherens junction proteins. Adhesion is conferred by calcium dependent homotypic interactions with cadherins in the adjacent cell membrane. Cadherins link indirectly to the actin cytoskeleton via β- or γ-catenin, which in turn links to α-catenin. This either connects directly with actin or via further proteins such as ZO-1. E-cadherin and N-cadherin, amongst other cadherins, have been shown to be present in the testis (Johnson et al., 2000).

Adherens junctions consisting of nectin-afadin-ponsin complexes are calcium independent and have been shown to associate with the cadherin-catenin complexes (Takai and Nakanishi, 2003).

Ectoplasmic specialisations (ES) are a modified type of adherens junctions found only in the testis. They are found between Sertoli cells, and Sertoli-germ cells, and have an important role in prevention of the premature release of elongate spermatids (reviewed in Mruk and Cheng, 2004). Espin is a component of apical and basal ectoplasmic specialisations, connecting to actin.

2.3.6.3 Gap Junctions

Gap junctions are a type of communicating junction. Gap junctions are intracellular channels which allow the transport of ions and small molecules (up to 1kDa), such as nutrients, second messengers and metabolite precursors, between adjacent cells (Pointis and Segretain, 2005). They consist of six integral membrane connexins, which make up a connexon. Two connexons (one from each cell) then make up the junction complex, with connexons often found clustered together in plaques. Connexin proteins have four transmembrane domains with both N- and C- terminals in the cytoplasm. Gap junctions are found between Sertoli cells and germ cells and adjacent Sertoli cells, and are thought to have a role in coordination of germ cell movement (reviewed in Mruk and Cheng, 2004). At least 20 connexin proteins are present in mammalian tissues, with connexin 43 the most important gap junction protein in the testis. Pelletier, 1995, showed that connexin 43 is present in the blood-testis barrier in a stage-dependent manner. The importance of connexin 43 in the testis was demonstrated by Brehm et al., 2007. They created a Sertoli cell specific connexin 43 knockout mouse, and showed that connexin 43 was required for the initiation of spermatogenesis, as well as a role in regulation of Sertoli cell maturation and proliferation, and that its role cannot be compensated for by other connexins (Brehm et al., 2007). However, Li et al., 2009a, showed that RNAi knockdown of connexin 43 in Sertoli cells in vitro did not cause any changes in the integrity of the tight junctions, suggesting that connexin 43 alone is not necessary for maintenance of BTB integrity.

2.3.6.4 Regulation of the Blood-Testis Barrier

Developing preleptotene/leptotene spermatocytes must traverse the blood-testis barrier at stage VII-IX of spermatogenesis in the rat. This requires disengagement of the junction complexes in the BTB; however, the barrier must still remain functional during this process so that the microenvironment of the adluminal compartment is maintained.

Unlike other blood-tissue barriers, tight junctions, adherens junctions and desmosome-like junctions coexist at the site of the blood-testis barrier. Yan and Cheng, 2005, investigated this unusual distribution of junction proteins and their role in regulation of blood-testis barrier dynamics. They proposed that under normal physiological conditions the tight junction and adherens junction proteins are engaged via interaction with their adaptor proteins (ZO-1, catenins). During restructuring of the blood-testis barrier to facilitate germ cell movement, there is a disengagement of the adaptor proteins and an increase in the levels of the tight junction and adherens junction proteins, suggesting that disengagement of the two types of junction proteins allows dissociation of the tight junction proteins at the BTB, allowing spermatocytes across the tight junction barrier, while the adherens junction proteins are still associated and temporarily supersede the tight junction barrier function and maintain the integrity of the BTB (Yan and Cheng, 2005). Similarly, a dissociation of adherens junctions to allow transport of the spermatocytes is accompanied by a surge in tight junction proteins to reinforce the tight junction barrier and maintain BTB integrity (Yan and Cheng, 2005).

Many signalling pathways have been implicated in restructuring of the blood-testis barrier. Cytokines, such as TNFα and TGF-β3 are thought to be secreted by primary spermatocytes and to have a role in induction of junction restructuring by causing accelerated endocytosis of the junction proteins and endosome-mediated degeneration leading to a reduction in the number of integral membrane junction proteins at the BTB (reviewed in Li et al., 2009b). Testosterone was also shown to enhance endocytosis of junction proteins at the BTB, however, it appears to promote recycling of the endocytosed proteins back to the cell surface rather than degeneration of the proteins, and could promote reassembly of the junctions during the restructuring process (Yan et al., 2008). IL-1α has also been shown to have a role in blood-testis barrier disruption, by acting on the actin cytoskeleton (reviewed in Li et al., 2009b). Gap junctions such as connexin 43 may also have a role in mediating BTB restructuring. Li et al., 2009a, suggested that a connexin 43/plakophilin-2 protein complex has an effect on redistribution of the tight junction proteins in the

BTB by affecting the phosphorylation state of the tight junction proteins (a loss of phosphorylation is thought to be related to a decline in the protein at the BTB) (Li et al., 2009a). Metalloproteases, hemidesmosomes and apical ectoplasmic specialisations have also been implicated in the regulation of BTB dynamics (reviewed in Li et al., 2009b). Proteases and protease inhibitors, as well as cAMP and intracellular phosphoprotein content have also been shown to have a role in blood-testis barrier dynamics (Lui et al., 2003).

2.4 The Pharmaceutical Industry

The pharmaceutical industry is a global business involved in the discovery, development, manufacture and marketing of drugs. In the UK, it takes an average of 10-12 years and £550million to develop a drug and bring it to market (Information and Statistics, ABPI website, http://www.abpi.org.uk/statistics/intro.asp accessed 14th January 2010). This long and expensive process involves many different stages and expertise, with essential criteria to meet at each stage to continue development of the drug. Drug discovery starts out with a target for a disease, such as a metabolic pathway thought to be altered in the disease state, and involves extensive investigations until lead drug compounds can be selected. The drug development process takes the lead compounds through stages from selection of the most promising candidate through clinical trials to launch of the drug. This process involves the development of drug formulations, investigations into the therapeutic doses, an assessment of drug safety, as well as analysis of drug metabolism. Following completion of these long processes, successful drug compounds are filed with the relevant agency and launched onto the market. Post-launch studies are carried out to ensure the drug is working as expected and to investigate any further manipulations that could be carried out so that the product could be re-launched with an alternative mechanism of dosing for example (Figure 2-6).

This project is in collaboration with GlaxoSmithKline (GSK), and therefore, the following sections will focus on practices at GSK, although the general pattern will

be observed by all pharmaceutical companies. GSK was formed in 2000 through the merger of SmithKline Beecham and Glaxo Wellcome, and is one of the leading pharmaceutical companies. GSK holds an estimated 7% of the world's pharmaceutical market (GSK literature). Research and Development (R&D) makes up a significant part of the company employing over 16,000 people, working at 24 sites in 7 different countries. GSK R&D has a leading position in genomics/genetics and new drug discovery techniques.

2.4.1 Safety Assessment

Ensuring that potential drug compounds will not cause adverse effects is an essential part of the drug development process. All drugs must be approved by the relevant agency, such as the US Food and Drugs Administration (FDA), before they can be launched commercially. Assessment of the safety of potential drugs is evaluated using non-clinical and clinical studies. Non-clinical safety testing involves in vitro and in vivo toxicology tests (general, reproductive and genetic), and safety pharmacology studies (detailed in Figure 2-6). Clinical tests involve administration of the potential drug compound to various groups of people in Phase I - IV trials (Table 2-1).

At GSK, the Safety Assessment division is involved in the non-clinical development of drug compounds, supporting the controlled administration to humans in clinical trials. UK Safety Assessment is divided into five departments, Pathology, General Toxicology, Investigative Preclinical Toxicology (IPT), Safety Pharmacology, and Genetic Toxicology.

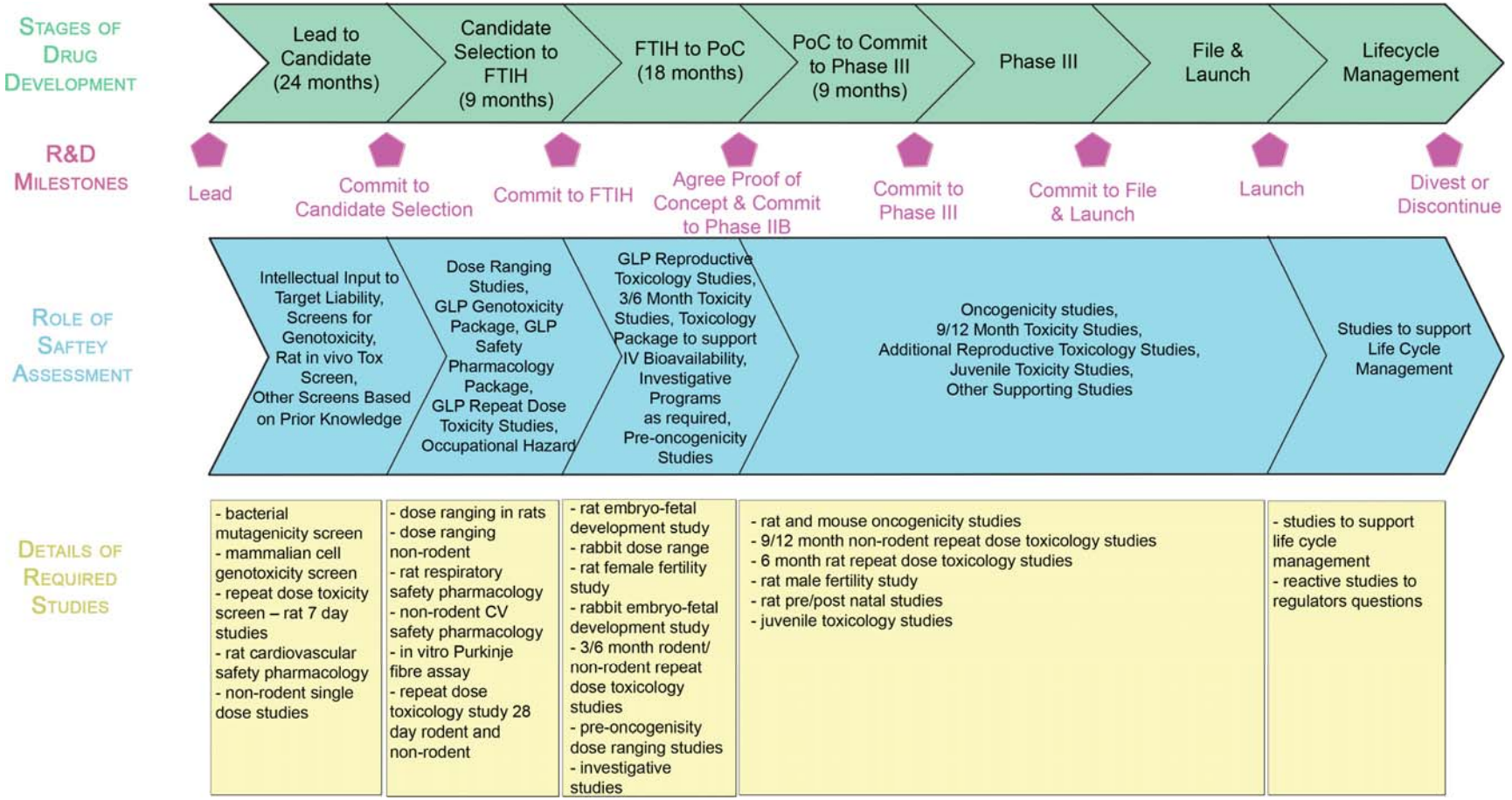


Figure 2-6 The drug development process, illustrating the relevant milestones in research and development of a drug. The role of Safety Assessment during the development stage is shown in blue, with more specific details of some of the studies required at each stage shown in the yellow boxes. FTIH – first time in human, PoC – proof of concept. *Adapted from GSK literature.*

2.4.2 Toxicology Testing

Non-clinical animal based toxicology studies are important in drug development for assessing the possible adverse effects a new potential drug compound may cause, prior to controlled administration to humans. Results of such studies can lead to the termination of development of a compound due to adverse effects or other limitations. Identification of problems as early in the process as possible is, therefore, essential.

Table 2-1 Clinical Trials for assessment of safety and efficacy of potential drugs *(information from ABPI publication – Clinical Trials – Developing New Medicines).*

Phase	Character of Trial
Phase I	Administration of drug to a small number of healthy volunteers
Phase II	Administration to a small group of patients (100-300) with the condition for which the drug has been developed
Phase III	Administration to a larger group of patients (1000-5000)
Phase IV	Post-licensing monitoring of the drug in the clinic

General toxicology studies involve the administration of varying concentrations of the compound to different animal models for different time periods. These studies are generally designed to directly support clinical trials (detailed in Table 2-1). The duration of the toxicology study is usually related to the duration and scale of the clinical trial. Generally studies are carried out in two species, one rodent, and one non-rodent, using doses of the drug exceeding the therapeutic dose. It is usual to try to show a no observed adverse effect level (NOAEL) or dose of compound that does not cause any adverse effects. Ideally this should be much higher than the intended human therapeutic dose. The number of studies that could be carried out is endless, therefore, suggested study guidelines are used to provide data to support clinical trials, with a consideration for the 3Rs.

The general drug development process, and suggested toxicology studies are shown in Figure 2-6. Usually a short initial study is conducted in a small number of rats,

prior to candidate selection, alongside genotoxicology screens and safety pharmacology screens. Following candidate selection, repeat dose studies are carried out for up to a month, to support first time in human studies (phase I). Longer repeat dose studies (3 – 6 months) are carried out to support the longer phase II trials, and proof of concept of the drug compound. At this point reproductive toxicology studies are also carried out if required (depending on the population group required for clinical trials). A female fertility study is carried out in the rat, alongside embryo-fetal development studies in the rat and rabbit. Investigative toxicology studies are also initiated to investigate any specific actions or adverse effects of the drug. Following proof of concept of the drug, further repeat dose toxicity studies are carried out (6, 9, 12 months in non-rodents). Oncogenicity studies are initiated, and male fertility studies and pre/post natal studies are also carried out, to support phase III clinical trials.

Species are selected for various reasons, including knowledge of the species, or their similarity to a human situation (for example pigs are used for dermal applications, and rabbits for embryo-fetal development). Route of administration is usually the intended route of administration in humans.

The exact toxicology studies carried out will depend upon the indication of the drug, how much is known about the class of drug, the route of administration, and the dose. An assessment of recovery of any adverse effects may be carried out, where animals are dosed for a set period of time, and then left for a recovery period before being examined for adverse effects.

Data obtained in toxicology studies must be carefully evaluated. The relevance of any adverse effects to humans and whether they may outweigh the therapeutic use must be carefully considered when interpreting results.

2.4.3 Detection of in vivo Toxicological Damage

Toxicity is assessed during the studies using a range of endpoints, depending on the exact aim of the study. Generally, all major organs are examined using histopathology, any observed clinical signs are recorded along with body weight changes and food consumption, and changes in clinical chemistry are assessed. This means that the organs are only examined at one point in time at the end of the study and may therefore not indicate if there have been any intermittent changes or whether there has been any damage that recovers during the study.

2.4.3.1 Biomarkers in Toxicology

Due to the increased cost and difficulty of developing drug compounds, it is becoming more and more important to be able to detect any potential adverse effects of drug compounds as early in the development process as possible. The importance of developing biomarkers to aid this is increasing.

Biomarkers are molecular indicators used to determine exposure and response to toxic chemicals. Biomarkers may be genes, proteins, small molecules or metabolites (Hale et al., 2003) and may be simple or complex, invasive (such as histopathology) or non-invasive (such as body weight) (Timbrell, 1998). Biomarkers should also be quantitative, sensitive, specific, easily measurable, relate to the biochemical mechanism, and work at realistic doses (Timbrell, 1998).

Common examples of biomarkers are the determination of prostate specific antigen for the early detection of prostate cancer (Koop, 2005), or the enzymes, aspartate transaminase (AST) and alanine transaminase (ALT) for the detection of hepatotoxicity (Timbrell, 2000). Proteomics, genomics and metabolomics are all being utilised for the discovery of biomarkers for toxicity (Hale et al., 2003; Suter et al., 2004; Koop, 2005). Biomarkers, especially genomics derived biomarkers, have the potential for detection early in a toxicological response, allowing indication of toxicity prior to major impairment. If used in drug toxicology studies, biomarkers

could help reduce the number of animals used, as the development of compounds could be terminated earlier than would otherwise be the case. This could also lead to a reduction in time and money spent on something that is not going to work, allowing the focus to change to other potential drug compounds. Biomarkers also have the potential to translate from non-clinical animal studies to clinical studies, to monitor any possible adverse effects.

2.4.4 The Testis as a Target Organ

The testes are an important target organ in non-clinical toxicology studies. They are the fourth most common target organ leading to attrition of potential drug compounds in GSK's non-clinical toxicology studies. Due to the nature of the drug development process, it is necessary to identify toxicity as early as possible in order to reduce the number of toxicology studies required before a compound is deemed to be unsuccessful. Specific biomarkers for testicular toxicity could improve the detection of adverse testicular effects by indicating testicular damage (changes to spermatogenesis and germ cell damage/degeneration) in early toxicity studies, possibly within hours of first exposure to treatment. Biomarkers could also have a role in understanding the onset, progression and recovery of testicular damage in non-clinical toxicology studies.

2.5 Testicular Toxicity

As described above, the testes are an important target for toxicity. The rest of this review will focus on an evaluation of well-studied testicular toxicants, which are used as compounds in investigations of testicular toxicity, and on current methods for the detection of testicular toxicity, with a review of previously investigated biomarkers for testicular toxicity, and an introduction to the investigative approach to be used in this thesis.

2.5.1 Testicular Toxicants

Well-studied testicular toxicants can be used as tools for investigation of testicular toxicity as well as in the elucidation of how different areas of the testis function (for example selective depletion of the Leydig cells could be useful in investigating the function and effects of the Leydig cells in vivo). Table 2-2 shows a range of cell specific toxicants which affect the male reproductive tract. One area of the testis that has not been well-characterised using specific testicular toxicants is the peritubular myoid cells, and little is known about their response to toxicological insult. A selection of well-known testicular toxicants will be discussed in more detail below, the Sertoli cell toxicants, dinitrobenzene, hexanedione, phthalate esters and cadmium chloride, the germ cell toxicant, methoxyacetic acid, and the Leydig cell toxicant, ethane dimethanesulphonate.

Table 2-2 Cell specific toxicants of the male reproductive tract. *Adapted from Creasy, 2001*

Target Cell	Toxicant	Effect
Leydig Cells	Ethane dimethanesulphonate	Leydig cell necrosis with secondary germ cell death and depletion and atrophy of secondary sex organs
	Lansoprazole	Inhibition of testosterone synthesis with secondary Leydig cell tumour induction
Sertoli Cells	Phthalate esters, 2,5-hexanedione, 1,3-dinitrobenzene	Sertoli cell vacuoles with secondary germ cell death and exfoliation
	Cadmium chloride	Disruption to tight junctions in blood-testis barrier, with a subsequent increase in fluid pressure and tissue ischaemia and necrosis
Spermatogonia	Busulfan, bleomycin	Spermatogonial cell death with secondary depletion of postspermatogonial cells
Spermatocytes	2-methoxyethanol, dinitropyrroles	Spermatocyte death with secondary depletion of postspermatocyte germ cells
Round spermatids	Ethylmethane sulphonate, methyl chloride	Spermatid death with secondary depletion of postspermatid germ cells
Elongated spermatids	Boric acid, dibromoacetic acid	Retention and phagocytosis of step 19 spermatids, abnormalities in released sperm

2.5.1.1 Sertoli Cell Toxicants

Sertoli cells are an important target for toxicants. Each Sertoli cell can only support a fixed number of germ cells so the number of Sertoli cells is directly related to sperm production, and due to the function of Sertoli cells, alterations may lead to impaired spermatogenesis, germ cell loss and infertility (Monsees et al., 2000). Toxicants can act on Sertoli cells in many different ways to induce testicular damage, including targeting germ cell-Sertoli cell interactions, disruption of the Sertoli cell cytoskeleton, disruption of Sertoli cell-Sertoli cell interactions at the site of the blood-testis barrier, alterations to receptors or second messengers, and alterations to nutrient supply (reviewed in Monsees et al., 2000).

The Sertoli cell toxicants, dinitrobenzene, hexanedione, phthalate esters and cadmium chloride will be discussed here. Each toxicant has a different mechanism of action. Further Sertoli cell toxicants include nitrobenzene (NB) (Allenby et al., 1990), carbendazim (Nakai et al., 1995), and cisplatin (Sawhney et al., 2005).

2.5.1.1.1 Dinitrobenzene

Dinitrobenzene is an organic aromatic compound, used in dye synthesis, manufacture of explosives, pesticides, and in the plastics industry (Reeve and Miller, 2002; McEuen et al., 1995). There are three isomers of dinitrobenzene, 1,2 (*ortho*)-dinitrobenzene, 1,3 (*meta*)-dinitrobenzene, and 1,4 (*para*)-dinitrobenzene. Studies reported in Blackburn et al., 1988, showed that 1,2-dintirobenzene had no toxic effects on rats, but that 1,3-dinitrobenzene and 1,4-dinitrobenzene both caused cyanosis and enlarged spleens. As well as these effects, 1,3-dinitrobenzene (referred to as DNB) was also shown to have an effect on the testis, suggesting a different mechanism of action for its testicular toxicity versus its effects on cyanosis and effects seen on the spleen (Blackburn et al., 1988). These results support previous studies reported by Watanabe et al., 1976, which showed that all three dinitrobenzene isomers (although 1,2-dintirobenzene to a lesser extent) cause methaemoglobinemia. This is the probable cause of the cyanosis reported by Blackburn et al., 1988. DNB

has been reported to be toxic to humans, following exposure through skin (Ishihara et al., 1976).

Linder et al., 1986, showed that high doses of DNB administered to male rats leads to infertility within 10 weeks due to diminished sperm quantity and quality, although following a 10 day recovery period, rapid regeneration of spermatogenesis was found, with complete recovery after 5 months. Single dose treatment of DNB led to degeneration of pachytene spermatocytes and round spermatids at stages VII-XII, with recovery of some seminiferous tubules between 16-72 days post-treatment, and full recovery 175 days post treatment (Hess et al., 1988). Blackburn et al., 1988, also reported damage to spermatocytes and spermatids, but observed this following vacuolation and retraction of Sertoli cells at stages VIII-XI following a single dose of DNB, suggesting a direct effect on the Sertoli cells. A further study carried out by Linder et al., 1988, with a single dose of DNB administered to adult Sprague-Dawley rats reported a decrease in testis weight, a decrease in number of testicular sperm 4 days after treatment and abnormal spermatozoa in the epididymis and a reduced number of motile sperm 16 days after treatment, reduced fertility 34 days after treatment, but some recovery after 175 days (Linder et al., 1988).

The testicular toxicity of DNB is thought to be due to a metabolite(s) of DNB. 1-nitroso-3-nitrobenzene (NNB) (or metabolites of this) is thought to be the toxic species following a reduction of one aromatic nitro group to form the amine (Cave and Foster, 1990).

The depletion of pachytene spermatocytes and round spermatids is thought to be due to apoptosis of the cells in a stage specific manner (Strandgaard and Miller, 1998; Muguruma et al., 2005). Strandgaard and Miller, 1998, reported that this apoptosis could be due to DNB acting on mitochondria in the Sertoli cells or the germ cells. This was further defined as an effect on the mitochondrial apoptosis pathway involving Bax and Bcl-2 rather than the Fas/Fas ligand pathway (Muguruma et al., 2005).

Reeve and Miller, 2002, reported that DNB acts on seminiferous tubule mitochondria, causing its effects by either oxidative stress through redox cycling of DNB to its nitroxyl anion radical, or by formation of NNB, as previously described above. NNB could bind to aldehyde dehyrogenase in the mitochondria and inhibit the reduction of NAD and oxidation of aldehydes (Reeve and Miller, 2002). The inhibition of aldehyde dehydrogenase has been shown to cause a reduction in cell number in a hepatoblastoma cell line, probably by forming protein adducts (Clemens et al., 2002). This suggests that DNB could be causing apoptosis of germ cells via its metabolism to NNB, which binds to aldehyde dehydrogenase in the mitochondria and leads to stimulation of the Bax/Bcl-2 pathway of apoptosis. DNB has also been shown to activate MAPK and JNK pathways (Lee et al., 2009).

2.5.1.1.2 Hexanedione

n-Hexane is an organic solvent used widely in industry, and is a constituent of gasoline (Ikeda and Kasahara, 1986). n-Hexane is metabolised to the γ-diketone, 2,5-hexanedione (HD) via P450 dependent hydroxylation and oxidation. HD combines with primary amines to form 2,5-dimethylpyrroles. 2,5-hexanedione has been shown to be toxic to both the nervous system and the testes, although each toxicity is caused by a separate mechanism (Boekelheide, 1987).

In the testis, HD exposure causes a reduction in ST fluid formation, followed by stage-specific Sertoli cell vacuolation and sloughing and loss of germ cells, leading to an altered spermatogenic cycle (Chapin et al., 1983). Boekelheide, 1988, reported adverse effects following administration of low dose HD for 2 weeks, with Sertoli cell vacuolation observed after 4 weeks and focal germ cell loss at 7 weeks. The mechanism of action of HD has been well-studied (reviewed in Boekelheide et al., 2003). The tubulin hypothesis was proposed by Boekelheide (Boekelheide, 1987) suggesting that HD alters microtubule assembly kinetics, which produces a change in the number and length of Sertoli cell microtubules which compromises Sertoli cell function, and in turn leads to disruption of germ cell maturation and testicular

atrophy. HD was shown to induce altered α-tubulin which fixes the tubulin heterodimers that make up microtubules into a pro-assembly conformation (Sioussat and Boekelheide, 1989). HD was also shown to have an effect on microtubule transport via its cross linking activity, and an altered distribution of microtubule associated motor proteins (such as dynein and kinesin) (reviewed in Boekelheide et al., 2003). The effect seen on the germ cells following HD treatment is thought to be due to the Sertoli cells sending an apoptosis signal to the germ cells they can no longer support following microtubule disruption.

Testicular effects of HD have been shown to be irreversible. The reasons for this are unknown, as the accumulated pyroles in the testis, and the Sertoli cell microtubule alterations do return to normal. Boekelheide, 1988, reported no increase in testis weight after a 17 week recovery period (12 cycles of the seminiferous epithelium), following low dose exposure for 2 weeks. Partial restoration of germ cell production was observed in 2/5 animals, with germ-cell depleted seminiferous tubules devoid of differentiating germ cells but containing spermatogonia (Boekelheide, 1987).

2.5.1.1.3 Phthalate Esters

Esters of phthalic acid are widely used as plasticisers (added to plastics to increase their flexibility). Several esters, including di(*n*-butyl) phthalate (DBP), dipentyl phthalate (DPP), di(2-ethylhexyl) phthalate (DEHP) and dihexyl phthalate (DHP) have been shown to have effects on male reproduction. In immature rats, this has been shown to be a direct effect on Sertoli cells causing Sertoli cell vacuolation and sloughing of spermatids and spermatocytes (Gray and Gangolli, 1986). The toxic effect of phthalates is due to metabolism or hydrolysis by esterases to phthalate monoesters (Li and Heindel, 1998).

Mature animals are less sensitive than immature animals to the adverse effects of phthalates, an effect thought to be due to differences in metabolism rather than in the Sertoli cells themselves. Species specific effects have also been observed, probably

for the same reason. Phthalates may alter energy metabolism by acting on the mitochondria (Creasy et al., 1987). The stage specificity of effects seen in adult testes also prompted investigation into the effects of phthalates on FSH. Phthalates are thought to decrease FSH binding and therefore reduce subsequent cAMP accumulation, which could be a further part of the mechanism responsible for their testicular toxicity (reviewed in Li and Heindel, 1998).

Over the past 30 years, many different mechanisms have been investigated in relation to phthalate induced testicular toxicity. These include compromise of zinc-dependent enzymatic activities, changes in hormonal status, altered metabolic function, disruption of Sertoli cell-germ cell interactions, and alterations in membrane dependent signalling (reviewed in Boekelheide et al., 2000).

Cater et al., 1977, investigated the effect of DBP treatment on zinc metabolism. They reported an increase in urinary zinc levels, due to changes in the uptake and turnover rate in the testis, probably caused by MBP or a metabolite of DBP acting as a non-specific chelator of divalent cations, leading to an increase in excretion and adverse effects in developing tissues (Cater et al., 1977). Foster et al., 1982, also looked at the effect of DBP treatment on zinc in rats. They observed a change in localisation of zinc, with a loss from the Golgi apparatus in the spermatids 6 hours after treatment, and a decrease in smooth endoplasmic reticulum localisation after 24 hours. They also reported a larger number of lipid bodies in the Sertoli and Leydig cells following DBP exposure, and Sertoli cell vacuolation (Foster et al., 1982).

2.5.1.1.4 Cadmium Chloride

Cadmium is a heavy metal, naturally found in trace amounts in water and air, and used industrially in a wide range of items such as pigments, alloys, electrical wire, plastics, rechargeable batteries, ceramic glazes and semiconductors (reviewed in Robards and Worsfold, 1991). Human exposure is from the industrial environment,

and via food, air, and water, although acute intoxication is rare (Robards and Worsfold, 1991).

Cadmium has been shown to be toxic to a range of organs including the kidney, prostate, liver, pancreas, ovary and testis. Cadmium toxicity in the testis was first shown by Alsberg and Schwartze, 1919, who observed blue testes following cadmium treatment (reviewed in Li and Heindel, 1998). Parizek and Zahor, 1956, observed severe damage to all testicular tissue 24-48 hours after administration of cadmium chloride to rats, with oedema of the interstitum and capillary stasis 2-4hrs after administration, regressive changes in the seminiferous epithelium after 4-6 hours and extensive haemorrhaging after 8 hours. Cadmium has been shown to cause toxic effects to the testes when administered to animals by sc or ip injection, but not when administered orally, suggesting that the route of administration is important. The effect of cadmium salts on the testis have been described in many species, and consist of disruption to the blood-testis barrier, germ cell loss, testicular oedema, haemorrhage, necrosis and sterility (reviewed in Siu et al., 2009).

Initial investigations into the mechanism of action of cadmium on the testes suggested that it increased vascular permeability. Aoki and Hoffer, 1978, reported formation of pores in testicular capillaries and venules leading to endothelial injuries, which promote interstitial oedema and haemorrhage, modifications in blood flow, and ischaemia. However, further investigations suggested that cadmium causes a loss of integrity of the blood-testis barrier, which leads to an increase in fluid pressure and tissue ischaemia and necrosis. Janecki et al., 1992, suggested that the tight junctions between Sertoli cells in an in vitro two-compartment culture system were disrupted following treatment with cadmium chloride. Hew et al., 1993a, showed that there was no apparent effect on the vasculature following a single low dose of cadmium chloride to male rats, but a stage specific failure of spermiation was observed, suggesting an effect on the Sertoli cells. The group went on to show that a single low dose of cadmium chloride resulted in stage-specific disruption of tight junction associated microfilament bundles at the basal region of the Sertoli cells,

providing further evidence for a direct effect of cadmium on the Sertoli cell tight junctions (Hew et al., 1993b).

Siu et al., 2009, reviewed current ideas about the mechanism of action of cadmium salts on the testis mediated by signalling pathways. The Cd^{2+} ion has similarities with Ca^{2+} and Zn^{2+} ions, and could substitute for them in crucial physiological processes resulting in activation or inhibition of signalling pathways (Figure 2-7). Siu et al., 2009, proposed a three phase system, starting with entry of Cd^{2+} ions into the Sertoli cells (or germ cells) via mechanisms such as diffusion, Ca^{2+} channels, the Cd^{2+}/Zn^{2+} channel, or Cd transporter ZIP8. Inside the cells, cadmium induces the synthesis and release of cytokines such as TGF-β3 which, via interaction with their receptors, activates the stress activated p38 MAPK signalling pathway. This pathway can disrupt the integral membrane proteins that make up the blood-testis barrier. The p38 MAPK pathway may also stimulate the production of proteases which leads to degradation of the integral membrane proteins (such as occludin, and cadherins) either via a direct effect of the proteases or via acceleration of clathrin-mediated endocytosis. Disruption of the Sertoli-Sertoli cell and Sertoli-germ cell junctions results in germ cell loss. Finally, in the last phase, to limit proteolysis, there is an activation of the c-JNK signalling pathway, which stimulates protease inhibitors (such as α_2-macroglobulin) to initiate recovery of the seminiferous epithelium (Siu et al., 2009).

Although the mechanism proposed by Siu et al., 2009, gives an insight into the mechanism of cadmium toxicity in the testis, the pathways have not been fully elucidated. Investigations using chemicals such as antioxidants (Kara et al., 2007; Manna et al., 2008; Acharya et al., 2008; Fouad et al., 2009) and calmodulin inhibitors (Martin et al., 2007) to prevent cadmium induced toxicity have suggested a role for oxidative stress in causing testicular damage, perhaps due to substitution of Ca^{2+} and Zn^{2+} ions in physiological processes. Further investigations into these effects, and the signalling pathways suggested by Siu et al., 2009, are required to unravel the exact mechanism/s involved in cadmium induced testicular damage.

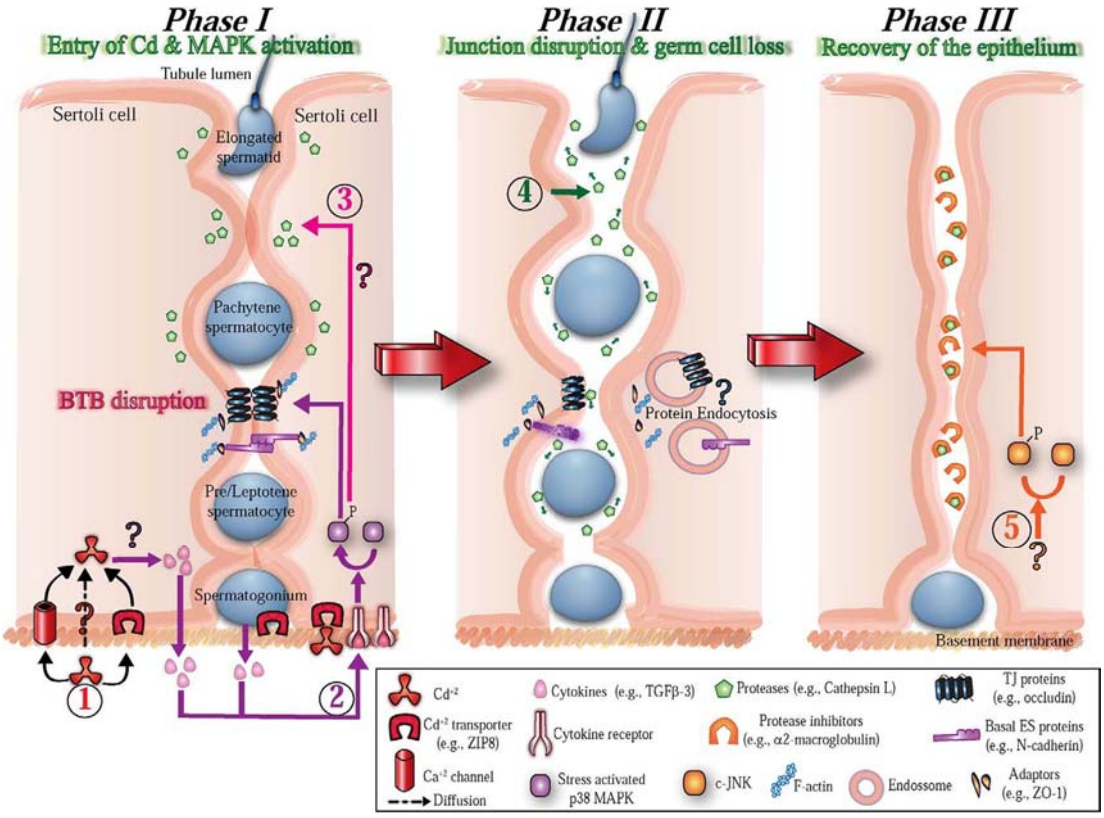


Figure 2-7 Proposed mechanism of action of cadmium salts on the testis mediated by signalling pathways via a three-phase system. Phase I: Cd ions enter Sertoli cells and/or germ cells via different mechanisms (step 1). Once inside the cells, Cd induces synthesis and release of cytokines which interact with their receptors and activate the stress activated p38 MAPK pathway (step 2) and can selectively disrupt the BTB. The production of proteases (possibly mediated by p38 MAPK) is also induced (step 3). Phase II: Disruption of the BTB via degradation of integral membrane junction proteins by proteases and/or the acceleration of clathrin mediated endocytosis which leads to germ cell loss (step 4). Phase III: Activation of the c-JNK signalling pathway stimulates production of protease inhibitors to initiate recovery of the seminiferous epithelium (step 5). *from Siu et al., 2009.*

2.5.1.2 Germ Cell Toxicants

Compared to the vast number of Sertoli cell toxicants studied, only relatively few germ cell specific toxicants have been described. The most commonly used is probably methoxyacetic acid which has a direct effect on the pachytene spermatocytes and will be discussed below. Further ways of inducing germ cell specific damage include x-irradiation (Haines et al., 2002). Mild scrotal heating can

also be used as a tool to cause disruption of the germ cells (Chowdhury and Steinberger, 1964).

2.5.1.2.1 Methoxyacetic Acid

Glycol ethers are used in a wide variety of products including inks, dyes, varnishes and as anti-icing agents in jet fuels. Ethylene glycol monomethyl ether (EGME) or 2-methoxyethanol (2-ME) is a commonly used glycol ether that has been shown to have toxic effects.

Metabolism of EGME is required for it to exert its toxic effect. EGME undergoes oxidative metabolism via alcohol dehydrogenase and aldehyde dehydrogenase to methoxyacetic acid (MAA) (Miller et al., 1982; Foster et al., 1984; Moss et al., 1985). EGME or MAA toxicity affects tissues with rapidly dividing cells and high energy metabolism, such as the testis, thymus and fetus.

EGME was shown to cause stage-specific cell death in the testis, affecting early and late stage pachytene spermatocytes (Creasy et al., 1985). A decrease in testis weight following repeated doses, flaccid consistency of the testes, and degeneration of the seminiferous epithelium was reported by Miller et al., 1982. Bartlett et al., 1988, showed a loss of pachytene and late zygotene spermatocytes 3 days following administration of MAA, at all stages except stage VII, shown by extracellular spaces at the site of the spermatocytes or collapsed epithelium, and a reduction in testicular weight, following a dose of 650mg/kg. Bartlett et al., 1988, also reported perturbation of Sertoli cells following the loss of a germ cell layer.

Moss et al., 1985, and Timbrell, 2000, suggest that the toxicity caused by MAA treatment is due to an effect on lactate production. Tirado et al., 2003, reported that the abnormal spermatogenesis caused by MAA treatment is due to a direct effect on the Sertoli cells (which may be in combination with a direct effect on germ cell activity), although they were not able to determine the mechanisms involved. Bagchi

and Waxman, 2008, reviewed the mechanisms of MAA induced spermatocyte apoptosis in relation to changes in gene expression and signalling pathways. They suggested that oxidative stress factors, protein kinases and nuclear receptors are all affected by MAA (Bagchi and Waxman, 2008). MAA induced apoptosis correlates with an increase in pro-apoptotic factors Bax and Bak compared to the pro-survival factor Bcl-W, triggering release of cytochrome C from mitochondria, leading to activation of caspases 9 and 3 which then leads to degradation of spermatocyte DNA. This process is thought to be induced by a disruption of the reduced:oxidised glutathione ratio due to oxidative stress (reviewed in Bagchi and Waxman, 2008) (illustrated in Figure 2-8). Bagchi et al., 2009, investigated the effect of MAA on androgen signalling, reporting that MAA potentiates the transcriptional activity of androgen receptor, and that it can regulate various intracellular pathways related to androgen synthesis. Wade et al., 2008, reported that MAA-induced cell death appeared to be associated with hyperacetylation of core histones H3 and H4 in the cells, following inhibition of HDAC, and activation of HAT, although the relation of this to apoptosis has not been elucidated. More recently, Tonkin et al., 2009, looked at the functional classification of gene expression changes following EGME or 2-ethoxyethanol (2-EE) treatment. They observed changes in the expression of genes related to protein kinase activity, the cell cycle, meiosis, protein transport and endocytosis. More specifically, increases in the expression of the transcription factor Wt1, and the actin binding protein, cortactin were reported (Tonkin et al., 2009). Cortactin is normally localised to the ectoplasmic specialisations and is involved in regulation of the actin cytoskeleton and endocytosis. Following EGME treatment, cortactin was localised to the spermatocytes suggesting a possible link between abnormal cortactin regulation and abnormal localisation and regulation of the tyrosine kinases that phosphorylate cortactin (Tonkin et al., 2009).

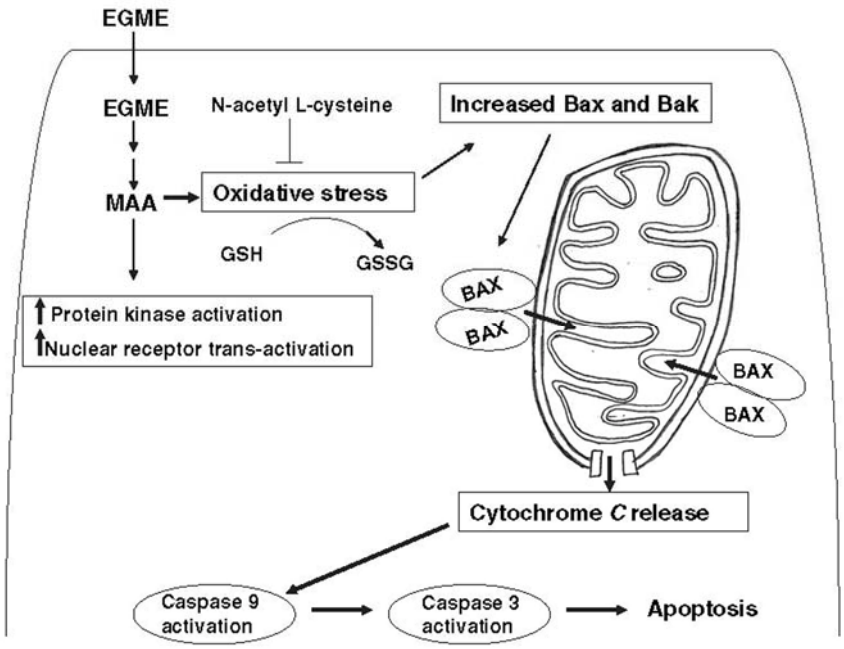


Figure 2-8 Schematic for proposed mechanism for MAA-induced apoptosis in spermatocytes. EGME is transported into the spermatocyte and oxidised to MAA, promoting an increase in protein kinase activation, nuclear receptor trans-activation and oxidative stress. Oxidative stress induces a disruption of the reduced:oxidised glutathione ratio, causing an increase in the apoptotic factors Bax and Bak, leading to the release of cytochrome c from the mitochondria. This activates caspase 9, and caspase 3 leading to apoptosis of the spermatocyte. *from Bagchi and Waxman, 2008.*

2.5.1.3 Leydig Cell Toxicants

Again, few Leydig cell toxicants have been described. The commonly used Leydig cell specific toxicant is ethane dimethylsulphonate.

2.5.1.3.1 Ethane Dimethanesulphonate

Ethane dimethanesulphonate (EDS) acts specifically on Leydig cells. Molenaar et al., 1985, showed a decrease in plasma testosterone levels 16 hours after EDS treatment of adult rats, with a further decrease between 16-48 hours post-treatment indicating a total loss of Leydig cell function. After 72 hours post-treatment, no Leydig cells were detected in the testicular interstitum, and effects on spermatogenesis and spermiation were observed 1 week after exposure, probably due to testosterone deprivation

(Molenaar et al., 1985). Repeat dosing of EDS over 6 weeks led to similar effects as the single dose treatment, then restoration to control levels of testosterone, suggesting that the effects on Leydig cells were reversible and that the regenerating Leydig cells are resistant to the effects of EDS (Morris, 1985). This effect could be due to the regenerating cells not being functionally mature so the target for EDS is not present (Morris, 1985).

The effects of EDS are both species specific (no effect on mouse Leydig cells) and age- specific (immature Leydig cells are not affected). The likely mechanism of EDS action is via apoptosis of Leydig cells, which has been shown both in vivo and in vitro, and could be due to depletion of glutathione due to EDS binding to glutathione (Morris et al., 1997).

2.5.2 Detection of Testicular Toxicity

Testicular toxicity can be detected by a variety of methods, each differing in their ease of use and sensitivity. These detection methods are important for analysis of male reproductive function in non-clinical toxicology testing as well as in studies investigating the effects of the well-known toxicants or proposed testicular toxicants.

2.5.2.1 Reproductive Organ Weights

The weights of the reproductive organs, including testes, epididymides, and prostate can be easily measured at the end of a toxicology study. Changes in weight following administration of a toxicant could indicate an effect on the organ. Morrissey et al., 1988, found that reproductive organ weights were likely to detect moderate reproductive toxicants in small groups of animals. An increase in organ weight following treatment would suggest an increase in fluid content in the tissue, whereas a decrease in organ weight would suggest decreased spermatogenesis (or decreased sperm content in the epididymides) (Creasy, 2003). However, following severe damage to seminiferous tubules, an increase in interstitial fluid volume can occur,

which can to some extent counterbalance any decrease in testicular weight following germ cell loss (Sharpe, 1988), although such an effect is most likely to be transitory and to lead eventually to a reduction in testis weight. A decrease in testis weight is still the most straightforward indicator of general testis damage.

2.5.2.2 Fertility

Assessment of male fertility carried out by administering the potential drug compound to the male throughout spermatogenesis and during mating with the female, provides an evaluation of effects on male reproductive function. This method is not very sensitive for the detection of specific and mild changes to spermatogenesis. Endpoints for these studies include: mating ratio, pregnancy ratio, pre- and post-implantation loss, litter size, the number of live and dead pups, sex ratio and offspring survival. These endpoints are relatively insensitive. For example, successful production of healthy offspring could be achieved even though damage to spermatogenesis has been caused, due to males releasing many more sperm than is required for fertilisation, so even a reduction in sperm production could still result in successful fertility (Amann, 1986). Fertility is, however, an important consideration in the evaluation of male reproductive toxicity, as it provides an evaluation of many aspects of sperm production/function as well as the reproductive tract and endocrine system.

2.5.2.3 Histopathology

Histopathology is the microscopic examination of tissues following their fixation and sectioning. It is an invasive technique but provides the chance to locate the exact area(s) of damage in a tissue. In the testis, the stages of spermatogenesis can be assessed to identify specific treatment effects such as missing germ cell layers or types, retained spermatids, multinucleated or apoptotic germ cells, and sloughing of germ cells into the lumen (Creasy, 2003). Histopathological examination of the testis has been described in the ICH guidelines (Safety Guideline S5(R2)) as the most

sensitive method for detection of drug effects on spermatogenesis. The importance of histopathology in the detection of testicular toxicity can also be gauged by the specific recommendations described by the regulatory authorities (summarised in Creasy, 2003) to maximise and improve detection of testicular toxicity. Histopathology is, however, time consuming and subjective, and also has the potential to miss adverse effects on the testis if only a limited number of tubules are evaluated (Suter et al., 1998a).

2.5.2.4 Sperm Analysis

ICH guidelines suggest that sperm analysis (sperm counts, motility and morphology) can be used as an optional way to confirm findings and further characterise effects observed with histopathological analysis, but are considered more relevant to fertility tests (ICH Safety Guideline S5(R2)). Sperm analysis is a non-invasive method of detection of testicular damage in many species, and can be used in both clinical and non-clinical studies. Many parameters such as sperm concentration, motility, vitality, and morphology, and homogenisation-resistant testicular spermatids and spermatozoa from the epididymis can be measured (Piffer et al., 2009). Sperm analysis provides a rapid, inexpensive and quantitative method for analysis of testicular damage in humans (Wyrobek et al., 1984). There are many variables in the analysis of sperm parameters, with different protocols set up in different laboratories. Guidelines on standardising the procedures have been discussed (Seed et al., 1996) in order to allow comparison of data from different sites. Sperm abnormalities, however, are unlikely to provide an early indication of testicular damage. Disruption leading to sperm abnormalities will have likely occurred at a much earlier time point in the spermatogenic cycle. Sperm analysis also provides no information on the genetic integrity of the sperm, an important consideration for reproduction (Rockett and Kim, 2005).

2.5.2.5 Hormones

The second function of the testis is the production of hormones and reproduction is controlled by the gonadotrophins (LH and FSH). Analysis of plasma hormone levels (LH, FSH, testosterone, Insl3 and inhibin B (refer to section 2.5.2.7.2)) could be used to evaluate testicular damage in combination with the techniques described above (as used in Piffer et al., 2009; Allenby et al., 1991). This method is, however, not commonly used in non-clinical toxicology studies, perhaps due to the invasive method of measuring hormone levels with repeated blood sampling required (Butterworth et al., 1995), and the inability to detect initial toxicant effects (such as those caused by HD treatment on Sertoli cells) (Chapin et al., 1982).

2.5.2.6 Flow Cytometry

Flow cytometry has been recently investigated as an invasive technique for use in the assessment of testicular damage. Suter et al., 1998b, investigated the sensitivity of the technique in comparison to histopathology following MAA treatment. Three parameter flow cytometry was carried out on enzymatically digested portions of testes. Their results suggested that there was no difference in sensitivity of detection of testicular damage caused by MAA treatment between flow cytometry and histopathology, and that flow cytometry could have advantages in the detection of testicular toxicity as it is quick, objective, and easy to carry out and requires only a small amount of tissue (Suter et al., 1998b). Oliveira et al., 2006, investigated the effect of cadmium chloride treatment on the testis using flow cytometry with fixed tissue samples. Significant alterations in the germ cell percentages were noted following cadmium chloride exposure (Oliveira et al., 2006). Flow cytometry would, however, need to be thoroughly investigated as a technique for detection of toxicity before it could be used in regulatory toxicology tests.

2.5.2.7 Potential for Biomarkers for Testicular Toxicity

Current techniques for evaluation of testicular toxicity such as sperm analysis and histopathology all have limitations. Biomarkers would be useful for the detection of testicular toxicity for early evaluation of male reproductive damage in toxicology studies, also for use as a high throughput procedure using a small amount of compound in early stages of drug development (discovery phase) (Yamamoto et al., 2005), and for longitudinal monitoring of testicular damage (Stewart and Turner, 2005). Successful biomarkers may also have a role in detection of testicular damage in clinical trials and in the clinic in general. Good biomarkers should be easily measured, and be robust, sensitive, quick and precise (Rockett and Kim, 2005).

At the moment, reliable markers of testicular damage are few and all are invasive, requiring blood or tissue samples (Timbrell, 1998). However, the unique nature of many of the proteins in the testis (testis specific proteins and testis specific isoforms) makes the testis a good source of potential biomarkers. Chemically-induced testicular damage can alter levels of proteins, the expression of which is regulated, directly or indirectly through the testis (Rockett and Kim, 2005). A small number of proteins have been previously investigated as potential biomarkers for testicular toxicity and will be discussed below; creatine, inhibin, lactate dehydrogenase-C4, and androgen binding protein. Other markers such as transferrin (Ber et al., 1990) and sperm surface protein SP22 have been shown to change in concentration in correlation with fertility (Rockett and Kim, 2005).

2.5.2.7.1 Creatine

Creatine is synthesised from arginine and glycine precursors in a two step reaction. This process is usually carried out in the liver and pancreas, but also occurs in the Sertoli cells (Moore et al., 1998). In the testis, creatine is thought to be further processed in the germ cells (Moore et al., 1998).

Creatine has been investigated as a non-invasive biomarker for testicular toxicity. Gray et al., 1990, showed an increase in levels of creatine in urine 48 hours following administration of cadmium chloride to adult male rats, and an increase in plasma creatine levels 24 hours following treatment. They also showed that these increases were due to the effect of cadmium chloride on the testis, rather than any other organ. However, they found that a loss of body weight could be contributing to the increased creatine levels rather than, specifically, the testicular damage (Gray et al., 1990). Draper and Timbrell, 1998, also observed increases in urinary creatine levels following cadmium chloride treatment, even at low doses where no pathology was observed. Moore et al., 1992, went on to further investigate the effects of specific testicular toxicants on urinary creatine levels. Treatment with MAA to disrupt the spermatocytes, and DNB or di-*n*-pentyl phthalate (DPP) to target the Sertoli cells, led to an increase in urinary creatine levels 24 hours after treatment (Moore et al., 1992). Treatment to affect the Leydig cells with EDS, however, had no effect on urinary creatine levels 24 hours after administration (Moore et al., 1992). All the studies described investigated creatine levels following a single dose of toxicant. Butterworth et al., 1995, went on to investigate the role for creatine in detection of chronic testicular damage by repeated dosing of EGME, and reported increased urinary creatine following repeated exposure.

Creatine is found in large amounts in muscle as well as the testis. Draper et al., 1994, investigated changes in urinary creatine concentration following administration of a specific muscle toxicant (2,3,5,6-tetramethyl *p*-phenylenediamine) to male rats, and noted an increase in urinary creatine following high dose treatment. However, they also reported testicular damage at this dose and suggest that this is the cause of the increased urinary creatine (Draper et al., 1994). Creatine levels in the urine have also been shown to increase following liver and heart damage (Gray et al., 1990). Timbrell et al., 1995, investigated using a combination of biomarkers, urinary taurine and creatine to enable distinction between increased urinary creatine due to testicular damage and liver damage. They proposed that the absence of changes in urinary taurine levels, in conjunction with an increase in urinary creatine would indicate

testicular damage, but a change in the levels of urinary taurine and an increase in urinary creatine would indicate liver damage (Timbrell et al., 1995).

2.5.2.7.2 Inhibin B

Inhibin B is a dimeric glycoprotein, made up of an α and a β_B subunit connected by a disulphide bond. The role of inhibin B in the male has recently been reinvestigated following the development of a two-site ELISA immunoassay to measure the concentrations of biologically active inhibin B, rather than the previously used 'Monash assay' which recognised only the α subunit of inhibin and therefore detected free α subunit forms as well as dimeric inhibin B (Anderson and Sharpe, 2000; Stewart and Turner, 2005).

Inhibin B has a role in regulation of FSH secretion via a negative feedback mechanism, with the ability to inhibit FSH but not LH production (Anawalt et al., 1996; Illingworth et al., 1996). Some investigations also suggest that inhibin is produced in the Leydig cells, although this is thought to have little impact on circulating concentrations of inhibin B (Anderson et al., 1998; Andersson et al., 1998). Other studies also suggest that the inhibin B β_B subunit could be produced in germ cells, although the mechanism for how the two inhibin B subunits would dimerise if produced in different cell types has not been proposed (Andersson et al., 1998).

Systemic concentrations of immunoreactive inhibin, especially inhibin B, have been broadly linked to spermatogenic status (reviewed by Stewart and Turner, 2005) as well as providing insight into changes in Sertoli cell function (Sharpe et al., 1988). Changes in the levels of inhibin B have been measured in a range of studies investigating human sperm concentration, and suggest a positive correlation between the two parameters, with a decrease in blood inhibin B levels in men with severe abnormalities of spermatogenesis (reviewed in Anderson and Sharpe, 2000). It has been suggested that this correlation is due to the role for inhibin B in the quantitative

feedback of the number of spermatozoa being released from the seminiferous epithelium, with phagocytosis of residual bodies by Sertoli cells contributing to inhibin B formation. This may also explain the proposed localisation of the β_B subunit to the germ cells (Anderson and Sharpe, 2000).

However, the sensitivity of changes in inhibin B following less marked effects on spermatogenesis is not clear, and does not appear to be as sensitive as histopathology (Stewart and Turner, 2005). An international collaborative project was set up to further investigate changes in inhibin following administration of different toxicants to rats, and the potential for inhibin B as a biomarker for testicular damage (Stewart and Turner, 2005), however the results have yet to be published.

2.5.2.7.3 Lactate Dehydrogenase-C4

Lactate dehydrogenase isozyme C4 (LDH-C4 or LDH-X) is specific to the testis and spermatozoa (Blanco and Zinkham, 1963; Zinkham et al., 1964), and is synthesised in a stage- and species- specific manner (Lalwani et al., 1996; Burgos et al., 1979). LDH-C4 has a role in glycolysis, in the NADH dependent reduction of pyruvate to lactate, in anaerobic conditions. Odet et al., 2008, disrupted the *Ldhc* gene in mice to investigate its role in the testis. Their results found *Ldhc*$^{-/-}$ males to be infertile, thought to be due to a progressive reduction in the motility of the spermatozoa, or due to defects in capacitation, all likely to be due to compromised ATP production due to the disruption to glycolysis (Odet et al., 2008).

Changes in the levels of plasma LDH-C4 have been utilised diagnostically for detection of testicular damage (Timbrell, 2000). LDH-C4 has been investigated as a marker for post-meiotic germ cell activity, with consistent positive correlations between LDH-C4 concentration in seminal plasma and sperm count observed (Gerezdeburgos et al., 1979; Orlando et al., 1988). However, some disagreement exists in the reported correlation of LDH-C4 levels and sperm motility (Gerezdeburgos et al., 1979; Orlando et al., 1988). LDH-C4 has also been

investigated in the development of new contraceptives, with evaluation of potential inhibitors of this specific isoform (Wong et al., 1997). Increased LDH-C4 plasma levels were observed following treatment with EGME and DNB (Reader et al., 1991). A slight increase in serum LDH-C4 levels was observed following high dose cadmium chloride treatment of rats (Draper and Timbrell, 1998), however the increase was not observed with lower doses, even though histopathological damage had occurred. This could be due to interference of the cadmium ions in the assay used (Draper and Timbrell, 1998).

2.5.2.7.4 Androgen Binding Protein

Androgen binding protein (ABP) is secreted bi-directionally from Sertoli cells. ABP levels in interstitial fluid in rats change during sexual maturation and have been shown to be increased in adulthood following treatments causing both stage- and germ cell- specific spermatogenic impairment, suggesting that serum and interstitial fluid ABP levels could be useful for in vivo monitoring of Sertoli cells (Sharpe and Bartlett, 1987). Suter et al., 1998a, reported an increase in testicular ABP levels following high dose DNB treatment of rats to induce Sertoli cell damage. Reader et al., 1991, also reported increased levels of plasma ABP levels, following germ cell or Sertoli cell damage (via treatment with either EGME or DNB). However, they suggest that the increased ABP levels may not reflect Sertoli cell damage, but may be due to disruption of spermatogenesis secondarily altering ABP secretion (Reader et al., 1991).

2.5.2.7.5 New Approach

A good biomarker should be easily measured, robust, sensitive, quick, and precise (Rockett and Kim, 2005). All the previously investigated biomarkers for testicular toxicity have limitations and have not been accepted for widespread use in the pharmaceutical industry. Therefore a new approach to finding biomarkers for testicular damage has been undertaken.

Sharpe, 1992, suggested that if you can identify proteins with key roles in spermatogenesis that find their way into the bloodstream, you could measure changes in their levels which might then give insight into disruption to particular steps in spermatogenesis. Following on from this, Turner et al., 1996, showed preliminary evidence that disruption of spermatogenesis by heat treatment, increased the number and amounts of seminiferous tubule-derived proteins in testicular interstitial fluid, and suggested that such proteins therefore have potential to be markers of disruption to spermatogenesis.

2.6 Aims of This Thesis

The main aims of this project were:

1. To investigate whether germ-cell specific proteins leak out of seminiferous tubules into interstitial fluid following toxicological insult, and if this principle could be applied to toxicants with unknown mechanisms of action.

2. To identify protein biomarkers that have the potential to signal an adverse change to spermatogenesis after toxic insult (or damage to the testes).

3. If parts 1 and 2 are successful, to develop a highly sensitive assay for detection of low levels of the protein biomarker in peripheral blood. This could be achieved using a technique such as fluorescent amplification catalysed by T7 polymerase technique (FACTT) (Zhang et al., 2006).

3 General Materials and Methods

3.1 Animal Work

Male Wistar rats were used for all studies (Harlan UK). Animals were maintained in our own animal facility, according to UK Home Office guidelines. Animals were housed in cages with a total of 6 adults/cage in a controlled environment. The rats had access ad libitum to water and a soy-free breeding diet (SDS, Dundee, UK). Where possible, young adult rats aged around 70-75 days, weighing approximately 300g, were used.

The 3Rs (reduction, refinement, and replacement of the use of animals in research) were considered when planning animal studies. The number of animals used was restricted to the smallest number possible to obtain reliable and accurate results.

3.2 Necropsy

Animals were killed according to UK Home Office guidelines using a rising concentration of carbon dioxide, followed by breaking of the neck. The bodyweights of the animals were recorded at necropsy.

3.3 Treatments

3.3.1 Cadmium chloride

Cadmium chloride (Sigma-Aldrich, Dorset, UK) was made up in 0.9% saline, at the required dose. The following doses were investigated; control (0mg/kg), 0.2, 1, 1.75, and 3 mg/kg. These doses were selected to show a dose response effect based on published literature (Hew et al., 1993b; Wong et al., 2004) and preliminary investigations (Section 5.2.1). Treatments were administered to adult male Wistar rats by i.p. injection at a concentration of 1mL/kg bodyweight. Tissue samples were collected approximately 24 hours after administration.

3.3.2 Methoxyacetic Acid

Methoxyacetic acid (MAA) (Fluka, Sigma-Aldrich, Dorset, UK) solutions were first adjusted for pH with concentrated sodium hydroxide to obtain a pH between the range pH 7.0-7.4, which is acceptable for dosing by oral gavage. Solutions were made up to the required volume with 0.9% saline. The following doses were investigated; control (0mg/kg), 200mg/kg, and 650mg/kg. These doses were selected based on a moderate effect previously seen at histopathological examination of the seminiferous epithelium with 650mg/kg MAA (Bartlett et al., 1988) and a mild effect previously observed with 200mg/kg MAA (Tirado et al., 2003; Wade et al., 2008). Treatments were administered to adult male rats by oral gavage at a concentration of 2mL/kg. Tissue samples were collected approximately 24 hours after administration.

3.3.3 1,3-dinitrobenzene

1,3-dinitrobenzene (DNB) (Sigma-Aldrich) solutions were prepared for dosing by oral gavage. In order to dissolve the solid, a small amount of DMSO (1.5%) (Sigma-Aldrich) was heated in a water bath, to aid dissolution. Solutions were made up to the required volume with corn oil. Solutions were vortexed to ensure complete mixing. The following doses were investigated; control (0mg/kg), 25mg/kg, and 50mg/kg. Doses were selected based on results published by Blackburn et al., 1988, where 50mg/kg DNB caused widespread damage to the seminiferous epithelium 24 hours after administration and 25mg/kg DNB caused mild damage. Treatments were administered to adult male rats by oral gavage at a concentration of 5mL/kg. Tissue samples were collected approximately 24 hours after administration.

3.3.4 Glycerol

A 15% glycerol solution was prepared by diluting glycerol (Fisher Scientific, Leicestershire, UK) with 0.9% saline. Glycerol treatments were administered by intra-testicular injection.

Rats were anaesthetised with isoflurane (in combination with air) in an anaesthesia box. Once under anaesthesia, rats were transferred to a mask for continued exposure to isoflurane during administration of the treatment. The inner (non-hairy) side of the scrotum was exposed by gently pushing the testes down tight into the bottom of the scrotum. 350µL 15% glycerol was slowly injected into the centre of the right testis using a 25G needle (based on methods investigated by Igdoura and Wiebe, 1994). 350µL 0.9% saline was slowly injected into the centre of the left testis using a 25G needle to act as a concurrent control. Animals were kept under observation while recovering from anaesthesia, and tissue samples were collected approximately 48 hours after administration.

3.3.5 Transforming Growth Factor-β3

Transforming growth factor-β3 (TGF-β3) (Calbiochem, Merck, Nottingham, UK) was reconstituted in 4mM HCl with 0.1% BSA (Sigma-Aldrich) according to the manufacturer's instructions. Reconstituted TGF-β3 was diluted to 1ng/µL in PBS. A control solution of 4mM HCl with 0.1% BSA diluted 1:10 in PBS was also prepared. TGF-β3 treatments were administered by intra-testicular injection, as in section 3.3.4, except that 200µL 1ng/µL TGF-β3 was injected at two sites in the right testis, 100µL/site. 200µL vehicle control solution was injected into two sites of the left testis (100µL/site). Administration of TGF-β3 treatment was based on the method published by Xia et al., 2006.

3.3.6 Biotin Tracer

EZ-link sulfo-NHS-LC-biotin (Pierce Bioscience, IL, USA) is a biotin conjugate. It can be used as a tracer to show whether the BTB is functioning, due to its' unique water soluble and membrane impermeable properties (manufacturers' literature, Tarulli et al., 2008). A 10mg/mL solution of the biotin tracer was prepared in 0.01M $CaCl_2$ in PBS. The solution was vortexed thoroughly to ensure the tracer was fully suspended. With adult rats, about 200µL of biotin tracer was injected by intra-

testicular injection into exposed testes of freshly-killed animals. Testes were then dissected out and left at room temperature for 30 minutes. With smaller testes from younger animals (day 10, 15, 18, 25), the testes were dissected out of the animals, and placed in a small volume of tracer solution for 30 minutes. Following incubation, testes were placed in Bouins fixative for the appropriate time (section 3.4.2.1).

3.4 Sample Collection

3.4.1 Peripheral Vein Blood

Immediately following necropsy, peripheral blood was collected by opening the chest cavity, and collecting blood from the heart using a syringe and a heparinised needle. Blood samples were kept on ice, and then centrifuged at 8000rpm for 15 minutes at 4^{o}C to separate cells from plasma. The plasma supernatant was pipetted into a clean tube and stored at -20^{o}C.

3.4.2 Preparation of Whole Testis Samples

3.4.2.1 Fixed Tissue

Testes were dissected out of freshly killed adult rats. All fat and connective tissue was removed from the outside of the testis. The testes were placed in Bouin's fixative (Triangle Biomedical Sciences, Lancashire, UK) for 3 hours. After this time, the testes were cut into 4 sections, and left to fix for a further 3 hours. After a total of 6 hours fixation, testes were placed in 70% ethanol (Fisher Scientific, Leicestershire, UK). Fixed testes were passed to the Histology support service for embedding in paraffin wax, using an automated Leica TP1050 processor (Leica Microsystems, Buckinghamshire, UK) and standard techniques. Testes collected from younger animals (day 10, 15, and 18) were fixed for a total of 4 hours, and left whole. For day 25 animals, testes were fixed for 6 hours and cut in half after 3 hours of fixation.

3.4.2.1.1 Sectioning of Fixed Tissue

Paraffin wax blocks embedded with fixed tissue were cooled on ice for at least 15 minutes. 5µm tissue sections were cut from the paraffin wax blocks using a manual rotary microtome (Leica RM2135). Sections were floated onto a water bath (45^{o}C) and mounted onto SuperFrost® glass slides (Menzel-Gläser, part of Thermo-Scientific, MA. USA). Slides were incubated at 50^{o}C overnight to complete the mounting process.

3.4.2.2 Snap-Frozen Tissue

Testes were dissected out of freshly killed rats. The capsule was removed from the testis, and remaining tissue squashed on aluminium foil and frozen in dry ice. Samples were stored at -80^{o}C.

3.4.3 Collection of Interstitial Fluid

Interstitial fluid was collected based on the method validated by Sharpe and Cooper, 1983. Testes were dissected out of freshly killed rats. Any remaining fat or connective tissue was trimmed, and the capsule carefully cut at the opposite end to the Rete testis, ensuring that no damage occurred to the underlying tubules. Testes were placed on ice, in pre-weighed 25mL Falcon tubes (1.5mL eppendorf tubes were used for smaller testes) containing 5µL protease inhibitor (Complete protease inhibitor, Roche, West Sussex, UK) at the bottom, with the cut end of the capsule facing downwards (Figure 3-1). Testes and tubes were weighed. Samples were left in the fridge (4^{o}C) overnight to allow interstitial fluid to drip out of the testes. Testes were then removed from the tubes, leaving as much fluid as possible in the tube. Interstitial fluid (IF) weights were taken. IF was removed to a 1.5mL eppendorf tube where appropriate, and centrifuged at 8,000rpm at 4^{o}C for 15 minutes. The supernatant was removed to a clean tube, and stored at -20^{o}C.

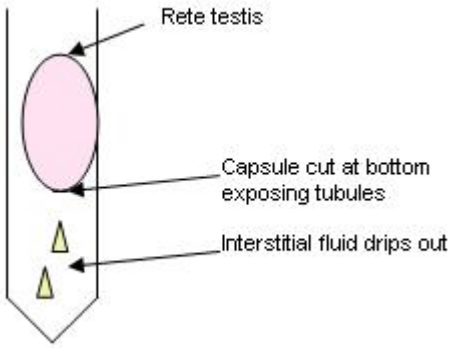


Figure 3-1 Collection of Interstitial Fluid. Each testis was placed in a tube with the capsule cut at the end opposite to the Rete testis and left overnight for the interstitial fluid to collect in the bottom of the tube.

3.4.4 Isolation of Seminiferous Tubules

Seminiferous tubules (STs) were isolated from freshly dissected testes. The capsule was removed from the testis. A few drops of PBS were added to aid dissection. Using a dissecting microscope, where necessary, each end of the testis was carefully pulled in opposite directions, exposing lengths of STs. The tubules were isolated, by securing each end of the section of tubule with forceps, and gently but firmly pulling upwards. Isolated tubules were collected, homogenised with RIPA buffer (section 3.12.7) containing protease inhibitor, and left to stand on ice. Then, the homogenised tubules were centrifuged at 13,200rpm at 4^{o}C for 10 minutes to remove any debris. The supernatant was removed to a clean tube and stored at -20^{o}C.

3.4.4.1 Isolation of Staged Seminiferous Tubules

Seminiferous tubules isolated as described above (section 3.4.4) were staged into 3 groups – I-V, VI-VIII, IX-XIV, under the dissecting microscope according to the pattern seen in the tubules (Figure 3-2), based on the method described by Parvinen and Vanhaper, 1972. Lengths of the same stage group were cut and pooled before homogenisation in RIPA buffer (as section 3.4.4).

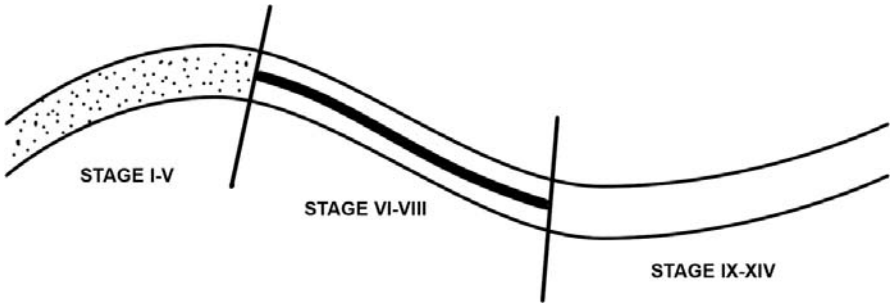


Figure 3-2 Staging Seminiferous Tubules. Tubules were dissected into three stage groups (stages I-V, VI-VIII and IX-XIV) according to the transillumination pattern seen in the tubule.

3.5 Immunohistochemistry

Immunohistochemistry was carried out on fixed tissue with a range of different antibodies (Table 3-1). The labelled streptavidin-biotin (LSAB) method was used. This is an indirect method of immunohistochemistry which amplifies the signal by using an unlabelled primary antibody, followed by a biotinylated secondary antibody and then a streptavidin-peroxidase conjugate (Figure 3-3).

Negative method control slides were used for each immunohistochemistry run, and incubated with normal serum instead of primary antibody. Positive control tissue (known to stain well for the selected antibody) was used when available to show whether the method had worked.

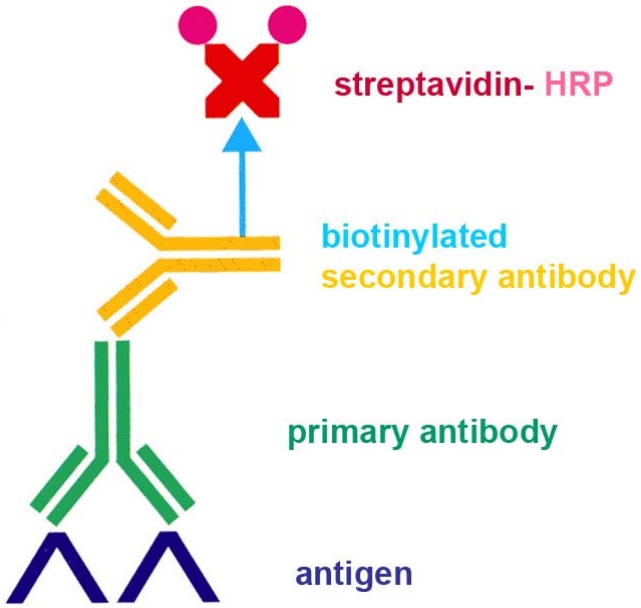


Figure 3-3 Schematic Diagram of LSAB immunohistochemistry. The primary antibody binds to the antigen on the tissue surface. A biotinylated secondary antibody raised to the species of the primary antibody binds to the complex. A streptavidin-HRP conjugate binds to the biotin on the secondary antibody. The HRP can then be detected using the chromagen DAB. *Adapted from Dako Handbook for Immunochemical Staining.*

3.5.1 Dewaxing and Rehydration of Tissue Sections

The first step in an immunohistochemistry run involves removal of wax from the fixed tissue sections. Slides were immersed in xylene for 4 minutes, and then placed in a fresh xylene bath for a further 4 minutes. This was followed by rehydration of the tissue by placing slides in 2 baths of absolute ethanol for 20 seconds in each, then 95% and 70% ethanol baths for 20 seconds each. Slides were finally placed in water.

3.5.2 Antigen Retrieval

Antigen retrieval or heat-induced epitope retrieval (HIER) was used for immunohistochemistry runs with certain antibodies (Table 3-1). This process is used to improve the immunoreactivity of antigens by breaking protein cross-links formed during the fixation process.

Buffer (either 0.01M citrate pH 6.0 buffer or 0.05M glycine pH 3.5 buffer) was heated to boiling in a pressure cooker. Slides in racks were then immersed in the buffer and the pressure cooker set to the highest pressure setting. Slides were heated for 5 minutes, then left to cool in the buffer for 20 minutes.

3.5.3 Wash Buffer

Tris buffered saline (TBS) was used to wash slides between each step. Typically slides were immersed in TBS twice, each for 5 minutes.

3.5.4 Blocking Steps

Two main blocking steps were carried out on all immunohistochemistry runs; a methanol/peroxide block, and a normal serum block. A streptavidin/biotin blocking step was included to reduce background staining for some antibody protocols. Blocking steps were carried out in a humidity chamber.

3.5.4.1 Methanol/Peroxide Block

Slides were incubated in a mixture of methanol (Fisher Scientific, ThermoFisher Scientific, MA, USA) and 3% hydrogen peroxide (VWR International, Leicestershire, UK) for 30 minutes. This blocking step was carried out to block endogenous peroxidase activity in the tissue in order to reduce background staining that could be caused by endogenous peroxidase reacting with the chromagen detection in the final step of the process.

3.5.4.2 Serum Block

In order to prevent non-specific binding of primary and secondary antibodies, a normal serum block with a low concentration of BSA was carried out. Slides were incubated with normal serum (Autogen Bioclear, Wiltshire, UK) with 5% BSA (Sigma) in TBS, for 30 minutes.

3.5.4.3 Streptavidin-Biotin Block

In order to reduce background with some antibody runs, a streptavidin-biotin blocking step was carried out. The Streptavidin/biotin blocking kit from Vector Laboratories (Peterborough, UK) was used. A drop of streptavidin solution was placed on each section for 15 minutes, to block any endogenous biotin or biotin-binding proteins in the tissue. Sections were washed, and then incubated with a drop of biotin solution for 15 minutes. This step is important in order to ensure all the biotin binding sites on the streptavidin molecules were occupied.

3.5.5 Primary Antibodies

Titre runs were carried out to work out the optimal concentration for each primary antibody. Antibodies were diluted to the required concentration (Table 3-1) in normal serum/5% BSA/TBS. Slides were incubated with primary antibody in a humidity chamber overnight at 4^{o}C. Appropriate negative control slides incubated without primary antibody were used.

Table 3-1 Primary Antibodies used for immunohistochemistry**.**

Primary Antibody	Dilution	Species Raised In	Antigen Retrieval	Streptavidin/ Biotin Block	Supplier
ADAM3	1:10	Mouse	Glycine pH3.5	No	Chemicon International[1]
Calpastatin	1:1000	Rabbit	Citrate pH6	No	Sigma
DAZL	1:500	Mouse	Citrate pH6	No	Serotec[2]
FABP9	1:10,000	Goat	Citrate pH6	No	R&D Systems[3]
STCM	1:500	Rabbit	none	No	N/A (see 4.3.1)
VASA	1:200	Rabbit	Citrate pH6	No	Abcam[4]

[1]Chemicon International, now part of Millipore, Herts, UK
[2]Serotec, Oxford, UK
[3]R&D Systems, Abingdon, UK
[4]Abcam, Cambridge, UK

3.5.6 Secondary Antibodies

Following overnight incubation with primary antibodies, biotinylated secondary antibodies were added. Secondary antibodies were chosen to be specific against the species in which the primary antibody was raised, and to be raised in the same species as the normal blocking serum used (Table 3-2). Biotinylated secondary antibodies were diluted 1:500 in normal blocking serum (5% BSA/TBS), and left on slides for 30 minutes.

Table 3-2 Secondary Antibodies used for immunohistochemistry.

Anti-species	Species raised	Conjugate	Supplier
Goat	Rabbit	Biotin	Vector Laboratories
Mouse	Rabbit	Biotin	Dako
Rabbit	Swine	Biotin	Dako

3.5.7 Chromagen Detection

Biotinylated secondary antibodies were detected using a streptavidin-horseradish peroxidase (HRP) conjugate. Streptavidin-HRP (Vector Laboratories, Peterborough, UK) was diluted 1:500 in TBS and applied to slides for 30 minutes.

The chromagen, 3,3'-diaminobenzidinetrahydrochloride (DAB) was then used to produce brown staining that could be visualised. DAB acts as an electron donor to the HRP, a process during which it becomes oxidised and produces the brown product, allowing visualisation of the location of the original tissue antigen.

DAB+ chromagen (Dako, Cambridgeshire, UK) was diluted in DAB+ substrate buffer, 1 drop:1mL as detailed in manufacturer's instructions, and placed on each slide until the staining had developed to the required intensity. The reaction was stopped by immersing slides in TBS.

3.5.8 Counterstaining, Dehydration and Mounting

Tissue sections were counterstained with haematoxylin nuclear stain. Slides were immersed in Harris' haematoxylin (Triangle Biomedical Sciences, Lancashire, UK) for 3-5 minutes (depending on whether antigen retrieval had been carried out), washed in water then rinsed in acid alcohol for 10 seconds. Slides were immersed in Scots Tap Water for 30 seconds then dehydrated by immersion through a series of increasing concentrations of alcohol solutions. Slides were placed in baths of 70%, 85%, and 95% ethanol for 20 seconds each, then two absolute ethanol baths for 20 seconds each. Finally slides were placed in xylene to replace the ethanol, in two separate baths for 4 minutes each.

Stained tissue was fixed to the slide by mounting suitable sized coverslips with Pertex. Slides were left to air dry.

3.5.9 Analysis of Stained Slides

Fixed stained slides were visualised using light microscopes. Micrographs of each slide were taken using a Provis Microscope (Olympus Optical, London, UK), fitted with a Canon DS126131 camera, and Canon EOS image capture software (Canon, Surrey, UK).

3.5.10 Detection of Apoptosis by TUNEL Immunohistochemistry

The terminal deoxynucleotidyl transferase-mediated (TdT) dUTP nick end labelling (TUNEL) method was used for looking at apoptosis in tissue sections. This is an in-situ method for detection of DNA fragmentation, by end-labelling of double stranded DNA breaks. The terminal transferase enzyme labels dsDNA breaks with digoxigenin (DIG)-dUTPs. The DIG-dUTPS are then detected with an anti-DIG antibody and then the general immunohistochemistry method is followed from the secondary antibody stage.

Following the methanol/peroxidase blocking step, slides were incubated with TdT and dig-11-dUTPs diluted in reaction buffer (all Roche, West Sussex, UK) at 37^{o}C in a humidity chamber for 30 minutes. Washes were performed in PBS, and then slides were incubated with normal rabbit serum for 10 minutes. Primary sheep-anti-DIG was diluted 1:50 in normal serum and placed on slides for 90 minutes. Following incubation with the secondary antibody, biotinylated rabbit anti-sheep (Vector Laboratories) at 1:500 in normal serum, TBS was used for washing steps. The protocol was completed using the Streptavidin-HRP and DAB detection system, as detailed above (section 3.5.7), followed by counterstaining and mounting (section 3.5.8).

3.6 Double Immunofluorescent Detection

Immunofluorescence was used to show the localisation (and co-localisation) of two antigens in the same tissue sections. This process uses the same basic principle as standard immunohistochemistry (section 3.5) but with fluorescent conjugated streptavidin or fluorescent amplification detection systems (tyramide).

This section details antibodies and tertiary reagents used for double immunofluorescent detection, as well as details on combined antibody protocols. Antigen retrieval and blocking steps were carried out as above (sections 3.5.2 and 3.5.4).

3.6.1 Wash Buffer

Phosphate buffered saline (PBS) was used to wash slides between each step. Typically slides were immersed in two baths of PBS, for 5 minutes each.

3.6.2 Primary Antibodies

As with basic DAB immunohistochemistry, titre runs were carried out to establish the optimal concentration for each primary antibody. Antibodies were diluted to the

required concentration (Table 3-3) in normal goat serum/5% BSA/PBS. Slides were incubated with primary antibody in a humidity chamber overnight at 4°C. Appropriate negative and method control slides were included in each run.

3.6.3 Secondary Antibodies and Detection Systems

Two different detection systems were used for immunofluorescent runs. The first method used biotinylated secondary antibodies (diluted in normal serum) and fluorescent labelled streptavidin conjugates (diluted in PBS) in the same way as the streptavidin-HRP detection system.

The second fluorescent detection method used the Tyramide Signal Amplification™ (TSA) system (PerkinElmer, MA, USA). This system is a quicker and more sensitive alternative to the fluorescently labelled streptavidin method. It works in a similar way, with HRP conjugated secondary antibodies used, followed by binding of flurophore labelled tyramide. Peroxidase labelled secondary antibodies were diluted in normal goat serum and incubated on slides for 1 hour. Tyramide-cyanine 3 or tyramide-fluroscein (PerkinElmer) was diluted 1:50 in TSA kit buffer, and incubated on slides for 10 minutes.

3.6.4 Counterstaining and Mounting

Dapi (Molecular Probes, Invitrogen, Paisley, UK) was used as a nuclear counterstain for immunofluorescent runs. Dapi was diluted 1:1000 in PBS and placed on slides for 10 minutes, keeping the slides in the dark as much as possible.

Slides were mounted with appropriate sized coverslips using PermaFluor™ aqueous mounting medium (ThermoScientific), and left in the dark at 4°C overnight to set.

3.6.5 Double Antibody Protocols

In order to carry out double immunofluorescent antibody protocols, the species that the primary antibodies are raised in must be considered. Ideally both antibodies should be raised in different species. However, two antibodies raised in the same species can be used with some modifications to the basic immunofluorescent protocol. Peroxidase labelled secondary antibody Fab fragments were used instead of whole IgG secondary antibodies. Fab fragments are much smaller than whole IgGs with only one binding site, allowing better antigen recognition and reduction in background signal. Slides were incubated with secondary antibody Fab fragments diluted in normal serum for 30 minutes.

A second antigen retrieval step was also carried out in situations where two same species primary antibodies were used. This step was carried out following tyramide detection of the first primary antibody. Slides were placed in boiling 0.01M citrate buffer (pH6) and heated in a microwave on full power for 2.5 minutes. Slides were then left to rest before continuing the protocol with a second serum blocking step, followed by incubation of the second primary antibody. Details of the protocols for double immunofluorescent studies are shown in Table 3-3.

Table 3-3 Double Immunofluorescent Protocol Information

All primary antibodies from Zymed, now part of Invitrogen, except anti- β-catenin, from BD Transduction Laboratories, Belgium.

Immuno	Primary Antibodies	dilution	Secondary Antibodies	dilution	detection	Citrate Antigen Retrieval	Avidin/Biotin Block
ZO-1/ occludin	ZO-1	1:100	Biotinylated goat anti-rabbit	1:200	Streptavidin -488	Yes	Yes
	occludin	1:100	Goat anti-mouse peroxidase	1:200	Tyramide-Cy3	No	No
Claudin-11/ ZO-1	Claudin-11	1:500	Goat anti-rabbit peroxidase Fab fragment	1:500	Tyramide-Cy3	No	No
	ZO-1	1:750	Goat anti-rabbit peroxidase Fab fragment	1:500	Tyramide-fluroscein	Yes	No
N-cadherin/ β-catenin	N-cadherin	1:14,000	Goat anti-mouse peroxidase Fab fragment	1:500	Tyramide-Cy3	Yes	No
	β-catenin	1:500	Goat anti-mouse peroxidase Fab fragment	1:500	Tyramide-fluroscein	Yes	No

3.6.6 Confocal Microscopy

Immunofluorescent slides were imaged using a Zeiss LSM510 Meta Confocal Microscope (Carl Zeiss Ltd, Hertfordshire, UK) and Zen software (Carl Zeiss Ltd).

3.6.7 Identification of Biotin Tracer

The biotin tracer (section 3.3.6) was detected using a direct immunofluorescent technique. Occludin was also stained for, to show the site of the blood-testis barrier, using a modified double immunofluorescent protocol.

Antigen retrieval (0.01M citrate, pH6) and blocking steps were carried out as detailed above (sections 3.5.2 and 3.5.4). Biotin tracer was detected by incubating slides with Streptavidin-488 diluted 1:200 in PBS for 1 hour. A second blocking step with normal goat serum was carried out for 30 minutes before addition of the occludin antibody. Detection of occludin was carried out using goat anti-mouse peroxidase and tyramide-Cy3 as detailed above (section 3.6.3). Slides were counterstained with Dapi and mounted with PermaFluor^{TM}.

3.7 Protein Assay

The total concentrations of protein present in IF and homogenised ST samples were determined using the BioRad DC Assay kit (Bio-Rad, Herts, UK). This is a colourimetric assay based on the Lowry protein assay, and uses a two step process. The first step involves the reaction between protein in the sample and copper in an alkaline solution. The second step involves the reduction of Folin reagent by the copper-treated protein. The reduced species have a characteristic blue colour which can be measured at a wavelength of 650-750nm, and compared to a protein standard curve to give the concentration of the sample protein.

Samples were first diluted in RIPA buffer containing complete protease inhibitor (Roche, West Sussex, UK) (a 1:50 dilution was sufficient for most samples). The Microplate Assay protocol was followed, according to the Manufacturers' guidelines. Plates were read at 690nm after developing the assay for 30 minutes. A BSA standard curve (diluted in RIPA buffer) was run on every plate to allow protein quantification.

3.8 Separation of Proteins by Gel Electrophoresis

Invitrogen's NuPAGE® System (Invitrogen, Paisley, UK) was used to separate proteins in IF and homogenised ST samples by size. The system utilises the traditional method of protein separation using polyacrylamide gels and electrophoresis with specially optimised buffers and pre-cast gels which allow increased gel stability, protein stability and consistent sharp band resolution.

3.8.1 Sample Preparation

In general, samples were prepared for loading 15µg protein in a total volume of 20µL. The required volume of sample was added to 5µL NuPAGE® 4x LDS Sample buffer (Invitrogen) and 2µL NuPAGE® 10x Reducing Agent (Invitrogen) to ensure complete reduction of proteins. Deionised water was added to make up the volume to 20µL. Samples were heated at 70^{o}C for 10 minutes to denature the proteins, and briefly centrifuged to collect the sample at the bottom of the tube, prior to loading onto the gel.

3.8.2 Gel Electrophoresis

Proteins were separated using 12% acrylamide 1mm NuPAGE® Novex bis-tris gels (Invitrogen). These gels allow denaturing gel electrophoresis, at a lower pH than conventional SDS-PAGE gels (Manufacturers literature). NuPAGE® SDS MOPS buffer (Invitrogen) was used as the running buffer, with NuPAGE® Antioxidant (Invitrogen) added to the cathode buffer chamber to prevent reduced proteins reoxidising during electrophoresis (Invitrogen literature). The SeeBlue Plus2 Pre-Stained Standard (Invitrogen) was run alongside samples on all gels, as a size marker. This standard contains 10 proteins, two with contrasting colours for easy band identification.

Gels were run at a constant voltage of 200V, for approximately 40 minutes or until the loading dye had reached the bottom of the gel.

3.9 Staining Gels for Total Protein

In order to look at all the proteins separated by gel electrophoresis, GelCode Blue Stain Reagent (Pierce, Thermo Scientific) was used. Traditionally proteins can be visualised on a gel using Coomassie Brilliant Blue Dye. GelCode Blue Stain Reagent is a Coomassie based gel stain, which utilises the properties of the Coomassie G-250 dye for protein staining on acrylamide gels (manufacturers' literature) and allows quantification of stained protein bands.

Gels were first pre-fixed with 50% methanol and 7% acetic acid for 15 minutes, due to running the gels in MOPS buffer. The gels were thoroughly washed, and then 25mL GelCode Blue Stain Reagent added, and incubated on a rocker at room temperature for 1 hour. Protein bands could be clearly seen to develop during the staining process. Gels were washed with ultrapure water for 1-2 hours to allow the stain to develop further, and remove any excess stain reagent.

3.9.1 Quantification of Protein Bands

GelCode Blue stained gels were scanned using ImageScanner III (GE Healthcare, Buckinghamshire, UK) and ImageMaster 2D Platinum software (GE Healthcare). Scanned images were saved as tif files for analysis with ImageQuant^{TM} TL software (GE Healthcare). Using the 1D Gel Analysis function of this software, protein bands of interest were manually selected in each lane by drawing boxes around them (Figure 3-4). The centre and edges of bands were automatically identified by the software using the pixel profile of the scanned gel (Figure 3-4). Band selection was manually adjusted to ensure only the band of interest had been selected in each box, and the rolling ball method of background detection chosen. This method allows a quantification of the total protein in each selected band based on the pixels, so that each band can be compared. A linear relationship between protein concentration and band intensity, calculated by this method, was shown to be accurate by running BSA standards on a gel, and quantifying the bands (Figure 3-5).

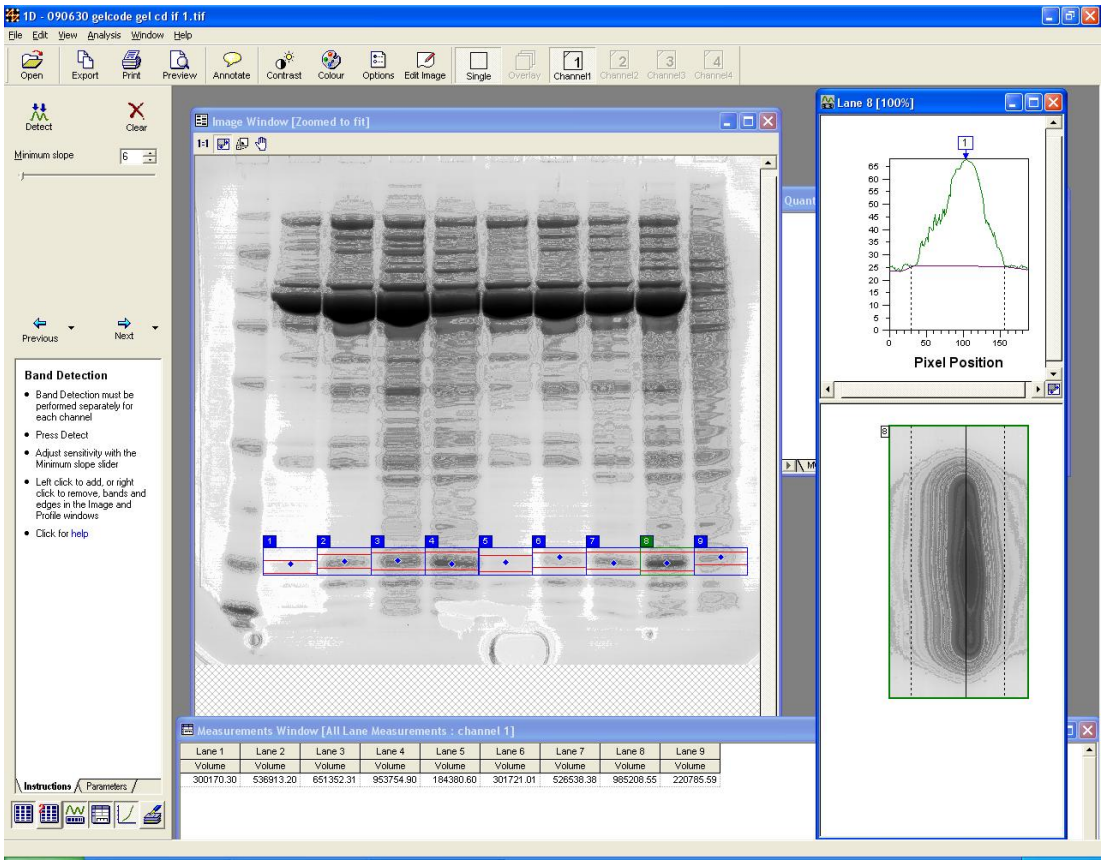


Figure 3-4 Screen-shot of ImageQuant™ TL Software used to quantify protein bands on 1D gels.

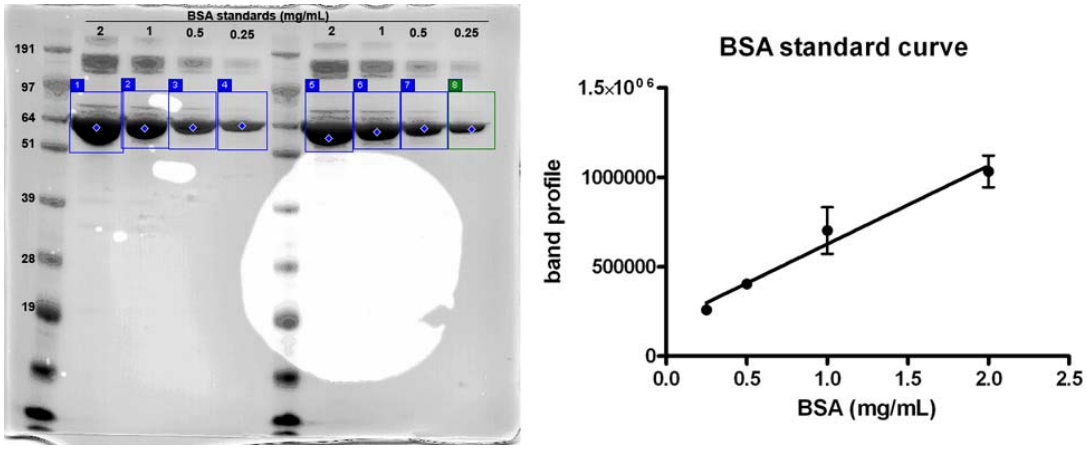


Figure 3-5 Validation of ImageQuant™ TL Band Analysis Technique.

In order to run all samples for comparison on gels, more than one gel had to be used. To allow a comparison of band profiles from two (or more) different gels, control homogenised ST samples were run on every gel, and bands normalised to the concurrent band in the ST sample (by expressing band profile values as a percentage of ST band profile value). Statistical analysis of results was carried out using a one way analysis of variance with a Tukey post-test, using GraphPad Prism software.

3.10 Western Blotting

Western blotting was used to look at specific proteins separated by gel electrophoresis. Proteins were transferred onto an Immobilon-FL PVDF membrane (Millipore, Herts, UK) for fluorescent detection, using a Hoefer TE22 transfer chamber (Amersham Biosciences, GE Healthcare, Buckinghamshire, UK). A 'sandwich' (Figure 3-6) was set up with gel and membrane in between layers of blotting paper, and sponges, surrounded by the blotting cassette. Individual components were equilibrated in transfer buffer before assembling the cassette. NuPAGE® transfer buffer (Invitrogen, Paisley, UK) (supplemented with NuPAGE® antioxidant (0.01%) and methanol (10%) according to manufacturers' instructions) was used for Western blotting. Up to four cassettes were placed in the transfer chamber, and transfer run for approximately 4 hours at 40V.

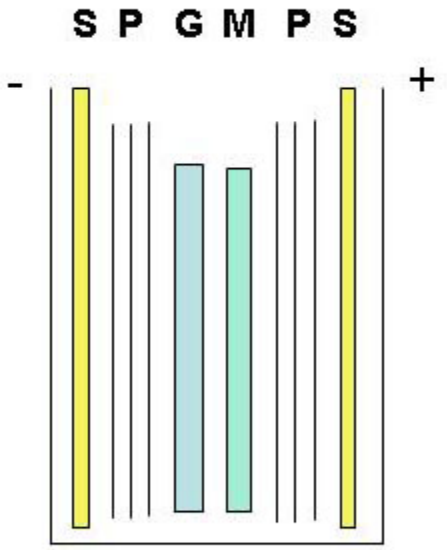


Figure 3-6 Western Blot Transfer Chamber showing 'sandwich' setup. Next to the cathode is a piece of sponge (S), 3x pieces of blotting paper (P), gel (G), membrane (M), 3x pieces of blotting paper (P), and a final piece of sponge (S) next to the anode.

Following transfer of the proteins to the Immobilon membrane, the membrane was blocked for 1 hour using one of two blocking buffers:

- Odyssey blocking buffer (Li-cor Biosciences, Cambridge, UK) diluted with PBS (1:1)
- Dried skimmed milk powder (20% w/v) in TBS+0.05% Tween-20 (Sigma)

Primary antibody was diluted to the required concentration (Table 3-4) in the same buffer, and incubated with the membrane overnight at 4^{o}C. Membranes were then washed using either PBS+0.1% Tween or TBS+0.05% Tween, depending on the blocking buffer used, then incubated at room temperature with fluorescent secondary antibodies diluted to 1:10,000 in blocking buffer for 1 hour (Table 3-5) The membrane was again washed, with a final wash without Tween.

Table 3-4 Primary Antibodies used for Western Blotting.

Antibody	Dilution	Supplier	Species Raised In
ADAM3	1:300	Chemicon International	Mouse
Calpastatin	1:1000	Sigma	Rabbit
DAZL	1:1000	Serotec	Mouse
FABP9	1:5000	R&D Systems	Goat
STCM	1:1000	N/A	Rabbit
VASA	1:1000	Abcam	Rabbit

Table 3-5 Secondary Antibodies used for Western Blotting.

Antibody	Supplier	Detection
Donkey anti-goat	Molecular Probes	680nm
Goat anti-mouse	Molecular Probes	680nm
Goat anti-rabbit	Rockland, USA	800nm

Membranes were analysed using the Odyssey Infrared Imaging System (Li-cor Biosciences). Blots were scanned, and viewed using the Odyssey Software (Li-cor Biosciences). Any bands identified on the blots were quantified by drawing boxes around the bands and measuring the intensity of the fluorescence (Figure 3-7).

As with 1D gels, in order to run all samples for comparison on Western blots, more than one blot had to be used. To allow a comparison of band intensities from two (or more) different blots, control homogenised ST samples were run on every blot, and bands normalised to the concurrent band in the ST sample (by expressing band intensities as a percentage of ST band intensity). Statistical analysis of results was carried out using a one way analysis of variance with a Tukey post-test, using GraphPad Prism software.

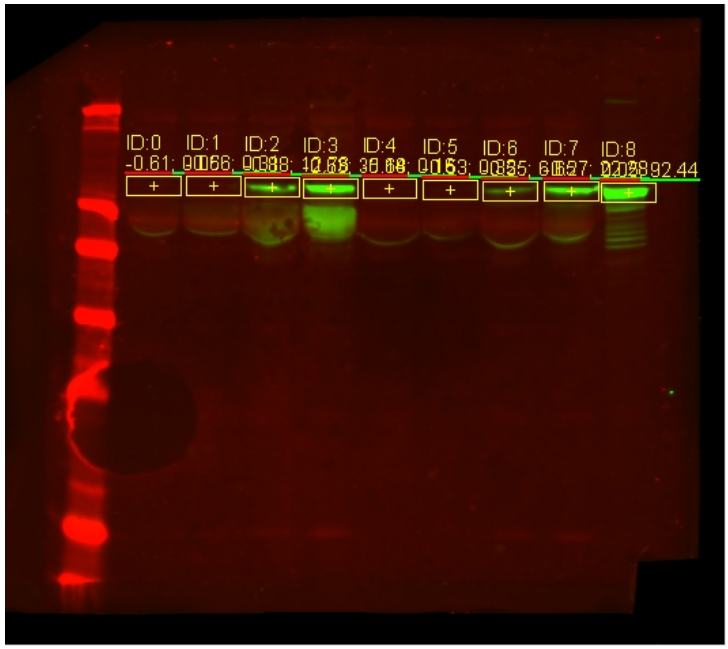


Figure 3-7 Western Blot Analysis using the Odyssey Infrared Imaging System and Odyssey Software. The intensity of each band was measured by drawing a box around it.

3.11 TaqMan® Q-PCR

3.11.1 RNA Preparation

3.11.1.1 RNA Extraction

RNA extraction was carried out using the Promega SV Total RNA Isolation kit (Promega, WI, USA). This kit uses a spin column assembly containing a silica membrane, to which the RNA, precipitated with ethanol, binds. Simple wash steps allow removal of proteins, salts and cell debris, while incubating with DNase I ensures the removal of contaminating genomic DNA.

Tissue was first homogenised in RNA lysis buffer (supplied with kit, Promega) using a tissue lyser (Qiagen, West Sussex, UK) and steel balls. The process was optimised to use 60-75mg frozen tissue, where possible (some testes from younger animals were below this range) in 450µL of lysis buffer, lysing for 1 minute at a frequency of 20 (1/s) in a deep well plate. RNA was isolated from the tissue lysates according to

the manufacturer's instructions, following the spin protocol, and performing two isolations per tissue lysate (resulting in 2 RNA samples/tissue sample).

Isolated RNA was analysed for integrity and quantity, and stored at -80^{o}C.

3.11.1.2 Analysis of RNA

3.11.1.2.1 RNA Integrity

The integrity of the RNA extracted from the testis samples was assessed using the Agilent 2100 Bioanalyser. This technique uses the 'lab-on-a-chip' technology to separate RNA by size, and using fluorescent detection with capillary electrophoresis. The Agilent RNA 6000 Nano kit (Agilent Technologies, Berkshire, UK) was used to prepare the chips (kit protocol followed). RNA samples were diluted 2:5 in DNase, RNase, and protease free water (5-PRIME, VWR International) before loading onto the chip. Results were assessed by analysing the gel/electrophoreograms produced and looking for the presence of only two peaks/bands representing 18S and 28S RNA (Figure 3-8).

The integrity of all isolated RNA samples was deemed to be suitable to continue with the PCR.

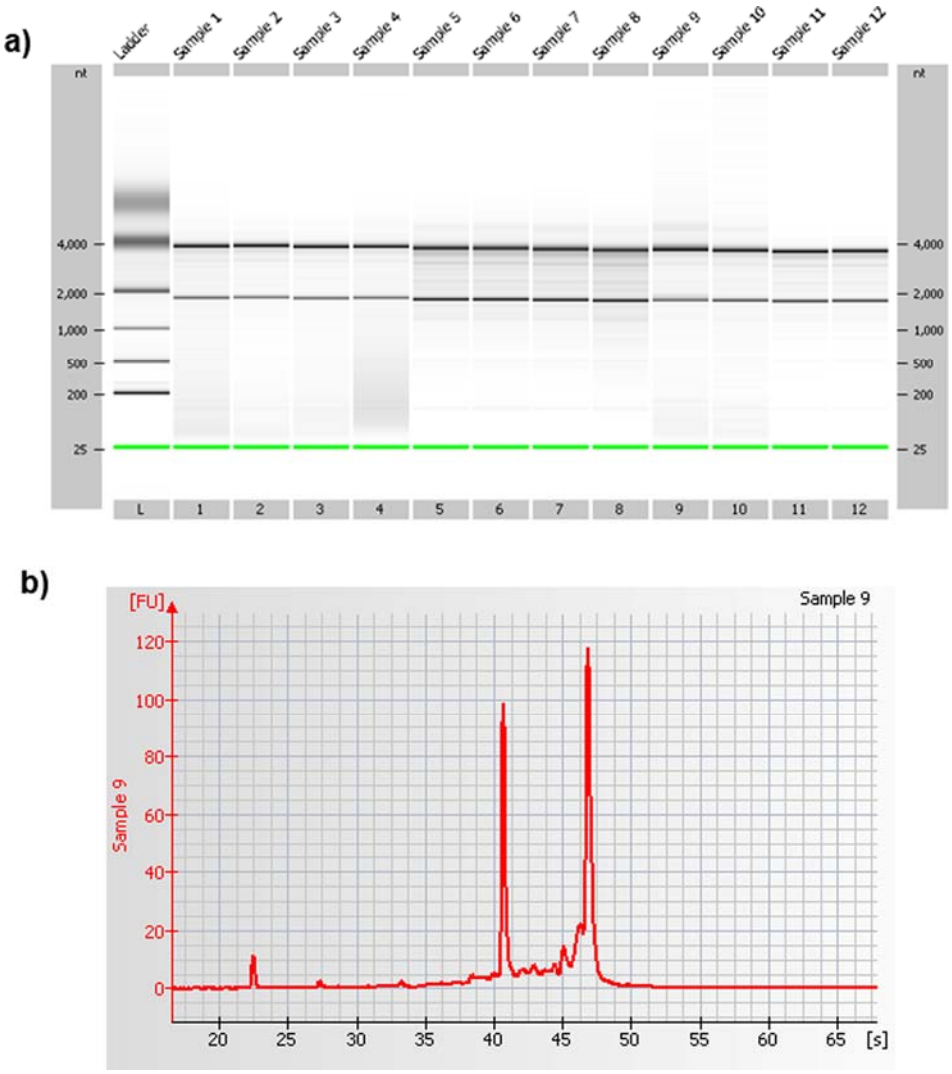


Figure 3-8 Example of read-out from Agilent 2100 Bioanalyser. RNA integrity results can be displayed as: (a) gel layout showing two clear bands for 18S and 28S RNA. Green band is marker band to allow alignment of all samples. (b) electrophoreogram layout showing two clear peaks for 18S and 28S RNA and a small marker peak.

3.11.1.2.2 RNA Quantity

The quantity of RNA extracted from the testis samples was evaluated using the ND-1000 Nanodrop (Labtech, East Sussex, UK). This is a spectrophotometer method which allows accurate quantification of a small volume of sample (2μL).

3.11.2 cDNA Preparation

cDNA was prepared using the Invitrogen Superscript ™ First-Strand Synthesis System for RT-PCR kit (Invitrogen, Paisley, UK) to synthesise cDNA from total RNA using random hexamers. This is a non-specific priming method for synthesising DNA. Reverse transcriptase (RT) positive and negative cDNA samples were synthesised from each RNA sample. RT minus samples were included to rule out any amplification of the genes of interest due to genomic DNA.

The volume required of each RNA sample was calculated, based on the assumption that 1µg RNA is converted to 1µg cDNA using the kit. A total of 3µg RNA was required for each RT+ and RT- samples, a total of 6µg RNA per sample. RNA samples that were too dilute for the kit protocol were spun down in a vacuum centrifuge, and then resuspended in the required volume of RNase/DNase free water. cDNA was synthesised according to the manufacturer's instructions supplied with the kit. cDNA was diluted to the required concentration of 0.01µg/µL, and stored at -20^{o}C.

3.11.3 TaqMan® Q-PCR

TaqMan® is the trademark name of the PCR-based, fluorogenic 5'-nuclease assay, which allows for the real time quantification of DNA. The assay uses a fluorogenic probe to enable the detection of a specific PCR product as it accumulates during PCR (Figure 3-9). A fluorescent reporter dye is incorporated on the 5' end, and a quencher on the 3' end of the probe. When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence. During PCR, if the target of interest is present, the probe specifically anneals between the forward and reverse primer sites. The 5' to 3' exonuclease activity of Taq polymerase cleaves the probe, increasing the proximity between the reporter and the quencher, causing an increase in fluorescence signal. The fluorescence at each PCR amplification cycle is recorded, allowing quantification of the accumulating PCR product.

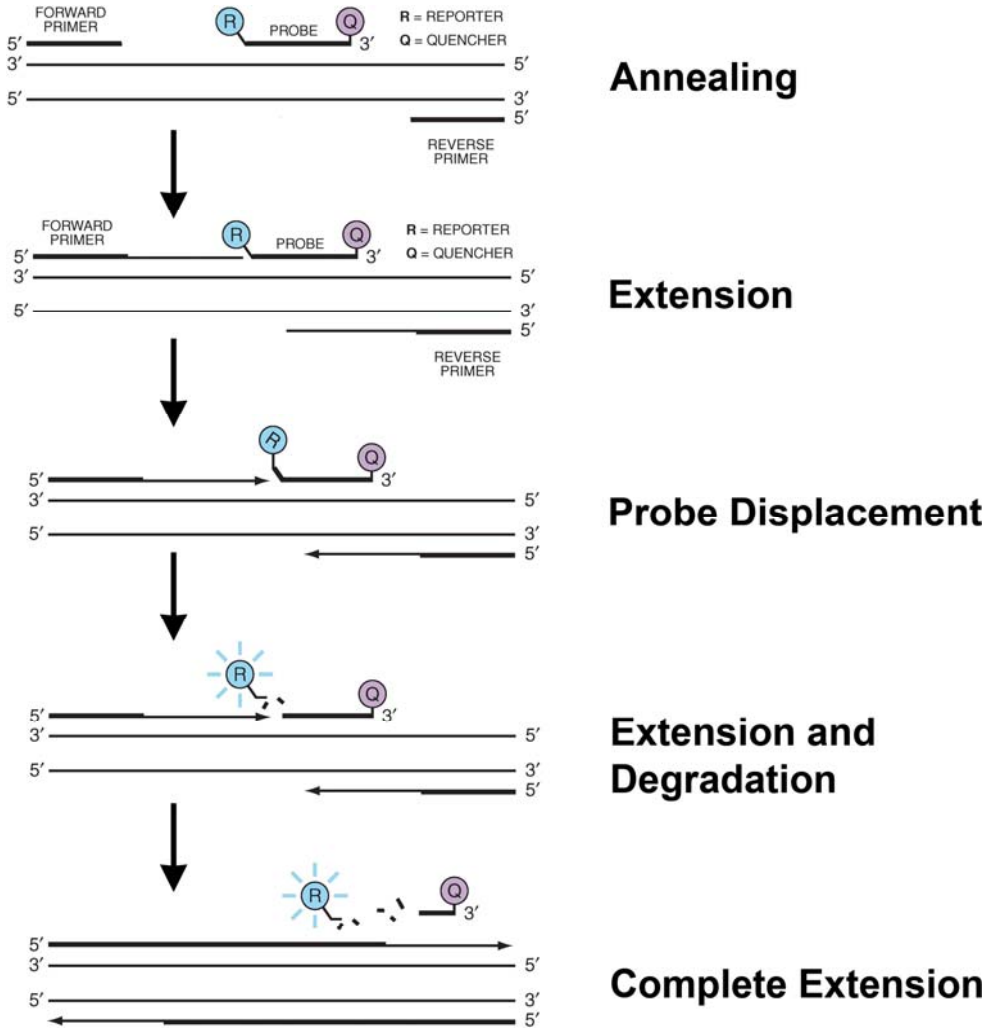


Figure 3-9 TaqMan® Q-PCR process. Forward and reverse primers bind to target sequence. Probe with reporter (R) and quencher (Q) dyes bind to DNA sequence between primers. 5'-3' exonuclease activity of Taq polymerase cleaves probe as it extends the primer sequences. Degradation of probe separates R and Q dyes causing an increase in fluorescence. Taq polymerase continues extension of PCR product. Amount of PCR product synthesised is proportional to the increase in fluorescence from the degraded probes. Adapted from Applied Biosystems website.

3.11.3.1 Primer and Probe design

Primers and probes were designed to eight genes of interest (3βHSD, β-catenin, claudin-11, FABP9, JAM-A, N-cadherin. occludin, ZO-1) using the Primer Express® software (Applied Biosystems, Roche). Rat sequences for the required genes were obtained, and entered into the software. TAMRA dye labelled probes were designed

for as many genes as possible, using the Applied Biosystems criteria. For genes, where no suitable primers and probes could be found (usually due to high G/C content in the probe), MGB probes were designed (Table 3-6). These probes are shorter than traditional probes with a non-fluorescent quencher and a minor groove binder at the 3' end. This increases the melting temperature of the probe, therefore allowing a shorter probe to be used, as well as increasing the difference in T_m between matched and mismatched probes. (Applied Biosystems literature). Primers and probes were tested using control cDNA before starting the main TaqMan® study.

Table 3-6 Primer and probe sequences for genes of interest and control genes. The Applied Biosystems kit was used for GAPDH. All other primers and probes were designed using Primer Express software, and supplied by Applied Biosystems.

Gene	Primer sequences	Probe type	Probe sequence
Occludin	F: GGAGAAGTGAGATAGAGGTGCATCT R: ACGAGAGTCCAAAGTCAGTTTGC	FAM-TAMRA	TCTACCTGCCCAGTGTCTGCAGTTCTCC
ZO-1	F: CTACCTTTGTATTCTCTCCTGTACCTCTT R: GACCTTATATGTGTAGAAATGCTGCTTT	FAM-TAMRA	AGCCTTGAACTTTGACCTCTGCAGCA
JAM-A	F: GTGGCTGTTAGTCACTTCATTTGG R: CACACATCTCCTCGATCCATATAAA	FAM-TAMRA	TGGAACTACTGCACAGACCCTTGTAA
Claudin-11	F: GCTGTTTAGCATAAGTTGTACTGTTGAA R: GCTTGCAGTGCCCAGAATCT	FAM-TAMRA	AACACCGATGGTCTGTCAGTGTCT
N-cadherin	F: AGGCTGCGAACCAGTTTGG R: CGGTGTTTTCCAGACAGTATCG	FAM-MGB	TCCCAGGGAATATCAGT
β-catenin	F: CAGGGAGAAGCCCTTGGATAT R: GCCGTATCCACCAGAGTGAAA	FAM-TAMRA	CCAGGACGATCCCAGCTACCGTTC
FABP9	F: TGGAGTGCACCATGAACAATG R: TCTACCCATCTGCTACACCCTTTC	FAM-MGB	TGTCAGCACTAGGACCT
3bHSD	F: TCCTGATGCCCTCTTTTGACA R: TGTGGACTAGCAAGGCTTCCA	FAM-MGB	AGACTCTAACTCTATGTCTTTA
18S	F: TTTTGTTGGTTTTCGGAACTGA R: CGGCGCAATACGAATGC	VIC-TAMRA	CCGGCCGTCCCTCTTAATCATGG
GAPDH	Applied Biosystems kit	VIC-MGB	
SPAG5	F: GCGGGCAGAGACAGAGACTAA R: GGGATCTACCTGGCCTTCCA	VIC-MGB	TGCTCCAGGAAGCC

Three housekeeping genes were selected to be used as controls. GAPDH and 18S are classic control genes used for PCR. SPAG5 was chosen as a previously validated testis specific control gene (Laura Robbins, GSK).

3.11.3.2 TaqMan® Q-PCR Reaction

TaqMan® reactions were carried out using clear 96-well plates (MicroAmp, Applied Biosystems). A "reverse plate" template was used. The plate layout was designed with all 11 genes on each plate, as well as an inter-plate control well, containing control cDNA and primers/probes for 18S (Table 3-7). Primers and probes were diluted to 10µM and 5µM, respectively, and mixed with RNase/DNase free water so that the required concentration (200nM) of each was reached in a total volume of 25µL, allowing 5µL primer/probe mixture to be loaded into each well. The PlateMate Plus (Matrix, ThermoScientific, Cheshire, UK) machine was used to stamp out all plates with 5µL primer/probe mix in each well, according to the plate layout. Plates were temporarily sealed with the ABgene ALPS-300 Plate Sealer (ThermoScientific) (106^{o}C) and stored at -20^{o}C until required.

Master mixes were prepared containing RNase/DNase free water, TaqMan® master mix (Applied Biosciences, Roche), and cDNA template (5uL 0.01µg/µL). 20µL of each mastermix was pipetted into each well of the stamped out plates using the Biomek FX (Beckman Coulter, High Wycombe, UK) automated machine. Mastermixes were pipetted along each row of the plates, allowing each cDNA template and gene combination, in duplicate. Plates were then sealed at 160^{o}C using the ABgene plate sealer and centrifuged at 1000rpm for 1 minute. Lids were placed on each plate, and the plates run overnight on ABI7900HT TaqMan® machines (Applied Biosciences, Roche).

Table 3-7 "Reverse Plate" Layout for TaqMan® Q-PCR. Grid represents each 96-well plate, stamped out with primers and probes for each of the 11 genes of interest down each column from 1-11. Well A12 contained primers and probes for 18S run with the same cDNA on each plate as an inter-plate control. Each cDNA sample was pipetted across a row on a plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	OCLN	ZO-1	JAM-A	claudin 11	N-cadherin	b-catenin	FABP9	3bHSD	18s	GAPDH	SPAG5	18s IPC
B	OCLN	ZO-1	JAM-A	claudin 11	N-cadherin	b-catenin	FABP9	3bHSD	18s	GAPDH	SPAG5	
C	OCLN	ZO-1	JAM-A	claudin 11	N-cadherin	b-catenin	FABP9	3bHSD	18s	GAPDH	SPAG5	
D	OCLN	ZO-1	JAM-A	claudin 11	N-cadherin	b-catenin	FABP9	3bHSD	18s	GAPDH	SPAG5	
E	OCLN	ZO-1	JAM-A	claudin 11	N-cadherin	b-catenin	FABP9	3bHSD	18s	GAPDH	SPAG5	
F	OCLN	ZO-1	JAM-A	claudin 11	N-cadherin	b-catenin	FABP9	3bHSD	18s	GAPDH	SPAG5	
G	OCLN	ZO-1	JAM-A	claudin 11	N-cadherin	b-catenin	FABP9	3bHSD	18s	GAPDH	SPAG5	
H	OCLN	ZO-1	JAM-A	claudin 11	N-cadherin	b-catenin	FABP9	3bHSD	18s	GAPDH	SPAG5	

3.11.3.3 TaqMan® Q-PCR Cycling Parameters

Amplification was performed according to the following program: 50˚C UNG activation step, a 95˚C AmpliTaq Gold enzyme activation, and 40 cycles of 95˚C denaturation and 60˚C anneal/extension (Figure 3-10).

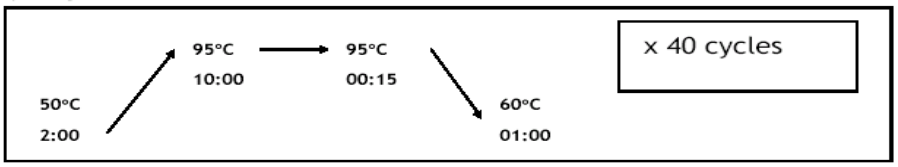


Figure 3-10 TaqMan® cycling parameters

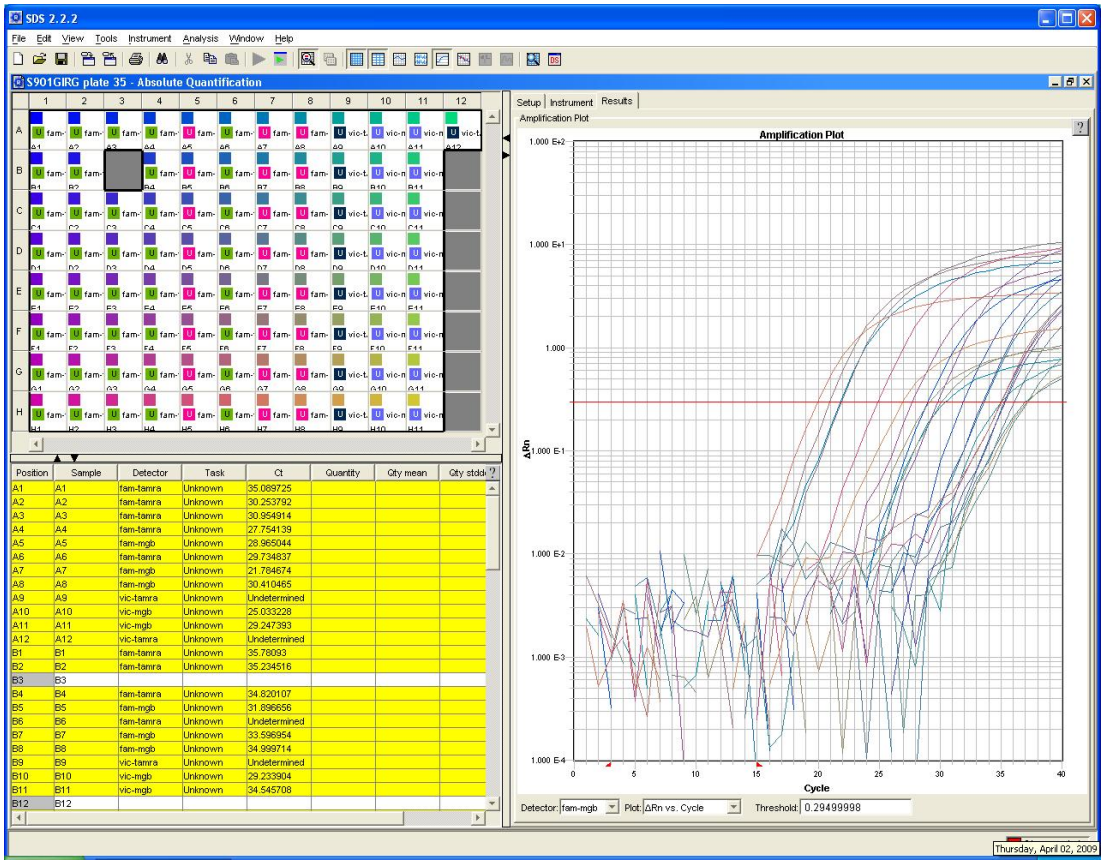
3.11.3.4 Standard curves

Rat genomic DNA (Clontech Laboratories, CA, USA) standards were made and run on the TaqMan® plates in triplicate alongside cDNA templates. Following the primer and probe test, cDNA standards were made up to run with β-catenin primers and probes as these did not work with genomic DNA.

3.11.3.5 TaqMan® Q-PCR Analysis of Results

Data analysis was performed using the threshold cycle (Ct) (the cycle at which amplification of the gene reaches exponential phase) (Figure 3-11). Using the TaqMan® SDS 2.2.2 software, a Ct value was selected to satisfy all genes. Each plate was checked, to ensure the selected value was suitable for all samples. Some samples were repeated due to insufficient results. Standard curves were produced from the genomic DNA (and cDNA for β-catenin) Ct values, and used to calculate copy numbers of each gene in each sample. The mean of the technical replicates of the samples from the TaqMan® plates was calculated, and RT- values subtracted from RT+ values to account for genomic DNA contamination. The mean of the values from each RNA extraction/tissue sample was then calculated to give a value for each tissue sample.

Some variability was observed with the data points, and those chosen to be out of the range of the data or those RT+ reactions that did not appear to work were excluded from the analysis. Results were analysed using log transformation and plotting mean values with SEM. Statistical analysis was carried out using one way ANOVA, followed by a Tukey post-test, using GraphPad prism software.



Position	Sample	Detector	Task	Ct	Quantity	Qty mean	Qty stddev
A1	A1	fam-tamra	Unknown	35.089725			
A2	A2	fam-tamra	Unknown	30.253792			
A3	A3	fam-tamra	Unknown	30.954914			
A4	A4	fam-tamra	Unknown	27.754139			
A5	A5	fam-mgb	Unknown	28.965044			
A6	A6	fam-tamra	Unknown	29.734837			
A7	A7	fam-mgb	Unknown	21.784674			
A8	A8	fam-mgb	Unknown	30.410465			
A9	A9	vic-tamra	Unknown	Undetermined			
A10	A10	vic-mgb	Unknown	25.033228			
A11	A11	vic-mgb	Unknown	29.247393			
A12	A12	vic-tamra	Unknown	Undetermined			
B1	B1	fam-tamra	Unknown	35.78093			
B2	B2	fam-tamra	Unknown	35.234516			
B3	B3						
B4	B4	fam-tamra	Unknown	34.820107			
B5	B5	fam-mgb	Unknown	31.896656			
B6	B6	fam-tamra	Unknown	Undetermined			
B7	B7	fam-mgb	Unknown	33.596954			
B8	B8	fam-mgb	Unknown	34.999714			
B9	B9	vic-tamra	Unknown	Undetermined			
B10	B10	vic-mgb	Unknown	29.233904			
B11	B11	vic-mgb	Unknown	34.545708			
B12	B12						

Figure 3-11 Screen-shot of SDS 2.2.2 Software for Analysis of TaqMan® Results. On the right hand side is the amplification plot. Different coloured lines denote the amplification curves of different samples. The threshold is shown by a red horizontal line, and the Ct is the cycle at which this line is crossed. A diagram of the 96 well plate is displayed in the top left hand corner of the screen. Below that is the TaqMan® data itself.

3.12 Commonly Used Buffers and Reagents

3.12.1 Tris Buffered Saline (10x working concentration)

605g Tris

876g NaCl

pH was adjusted to 7.4 using HCl (at least 300mL required)

make up to 10L with deionised water

3.12.2 Phosphate Buffered Saline

Dissolve PBS tablets (Sigma-Aldrich) 1 in 20mL deionised water

3.12.3 Citrate Buffer (0.1M)

42.02g citric acid

1900mL distilled water

Adjust to pH6.0 with concentrated NaOH

Make up to 2L

3.12.4 Glycine Buffer (0.5M)

75.07g glycine

1900mL distilled water

Adjust to pH3.5 using concentrated HCl

Make up to 2L

Add 2g EDTA

3.12.5 Scots Tap water

10g potassium hydrogen carbonate

100g magnesium sulphate

5L tap water

3.12.6 Acid Alcohol

70% ethanol

1% concentrated HCl

3.12.7 RIPA Buffer (5x working concentration)

750mM NaCl

250mM TrisHCl pH7.4

25mM EDTA

5% sodium deoxycholate

0.5% SDS

3.12.8 Homogenisation buffer

For 5mL

3.5mL deionised water

0.5mL 10x Complete protease inhibitor

1.0mL 5x RIPA buffer

3.12.9 TUNEL Reaction buffer

30mM TrisHCl pH7.2

140mM Na Cacodylate

1.5mM CoCl

3.12.10 Bouin's Fixative

Prepared by Triangle Biomedical Sciences (Lancashire, UK)

1L 40% formaldehyde

200mL glacial acetic acid

4L saturated picric acid

4 Normal Testis and Investigative Tools used in this Thesis

4.1 Introduction

Throughout this thesis various testicular toxicants with differing target areas have been used. Before tissues collected from animals given these treatments can be evaluated, it is important to understand the tissue, and how it responds in different experimental conditions. This chapter aims to introduce the different approaches, techniques and tools used in this thesis with vehicle control samples in order to understand the 'normal' situation.

The aim of this project is to evaluate the potential for biomarkers for testicular toxicity. Previous work (Turner et al., 1996) suggested that proteins may leak from seminiferous tubules into the interstitum under toxicological insult to the testis. This could be due to either loss of integrity of the blood-testis barrier, or germ-cell damage. In order to investigate this, adult male Wistar rats were treated with testicular toxicants with differing targets of action. Analysis of germ cell damage, blood-testis barrier integrity, and an evaluation of proteins in interstitial fluid samples were carried out on samples collected from the treated animals.

As well as the challenge of working with a complex tissue, in order to evaluate potential biomarkers, it is necessary to consider a large number of proteins. Three approaches were taken to investigate potential biomarkers; a general antibody to seminiferous tubule proteins was used to look at a range of proteins, 1D Coomassie gel analysis was used to assess total proteins in samples, and specific proteins were selected from an Affymetrix Genechip® Screen carried out at GSK as potential biomarkers for testis toxicity.

This chapter introduces these three approaches as well as detailing methods used for evaluation of blood-testis barrier integrity.

4.2 Methods

4.2.1 Samples

Studies were set up to investigate known testicular toxicants. A group of vehicle control treated animals were used for comparison with each study. The results in this chapter all used fixed testes or interstitial fluid samples from vehicle control treated animals for these studies. Isolated and staged seminiferous tubules (refer to section 3.4.4.1) were also used in this chapter.

4.2.2 Immunohistochemistry and Immunofluorescence

Immunohistochemistry with antibodies to the proteins of interest (DAZL, VASA, ADAM3, Calpastatin, FABP9), and a general antibody raised to seminiferous tubule conditioned medium, was carried out to investigate the localisation of proteins in the testis. The DAB protocol described in section 3.5 was used.

Immunofluorescence was used to investigate the blood-testis barrier proteins, ZO-1, occludin, claudin-11, N-cadherin, and β-catenin. Immunofluorescent detection was also used to detect the biotin tracer. Detailed protocols are described in chapter 3.

4.2.3 Coomassie Stained Gels and Western Blotting

Proteins in IF and ST samples were separated using 1D gels. Coomassie stain was used to look at total protein, and Western blotting with the STCM antibody, and an antibody to FABP9 were carried out following the methods described in sections 3.9 and 3.10.

4.3 Results

4.3.1 Antibody raised to Seminiferous Tubule Conditioned Medium

An antibody raised to seminiferous tubule conditioned medium (STCM) was prepared by a previous PhD student (Katie Turner, Turner, 1996). Seminiferous tubules were isolated from adult rats and cultured. Conditioned medium was collected and pooled from 4 rats, before being lyophilised, reconstituted and emulsified. Two rabbits were injected with the resulting immunogen at five different intradermal sites, followed by booster injections nine weeks later, and collection of blood samples after a further 3 weeks. The IgG fraction was prepared from the serum by affinity chromatography producing the polyvalent antiserum to STCM (Turner, 1996; Turner et al., 1996).

The antibody detects a range of seminiferous tubule derived proteins as shown in Figure 4-1, although the exact identity of these proteins is unknown. The antibody was used in immunohistochemistry and Western blotting to investigate general changes in the proteins present in control and treated testis samples, in initial investigations into the protein leakage hypothesis.

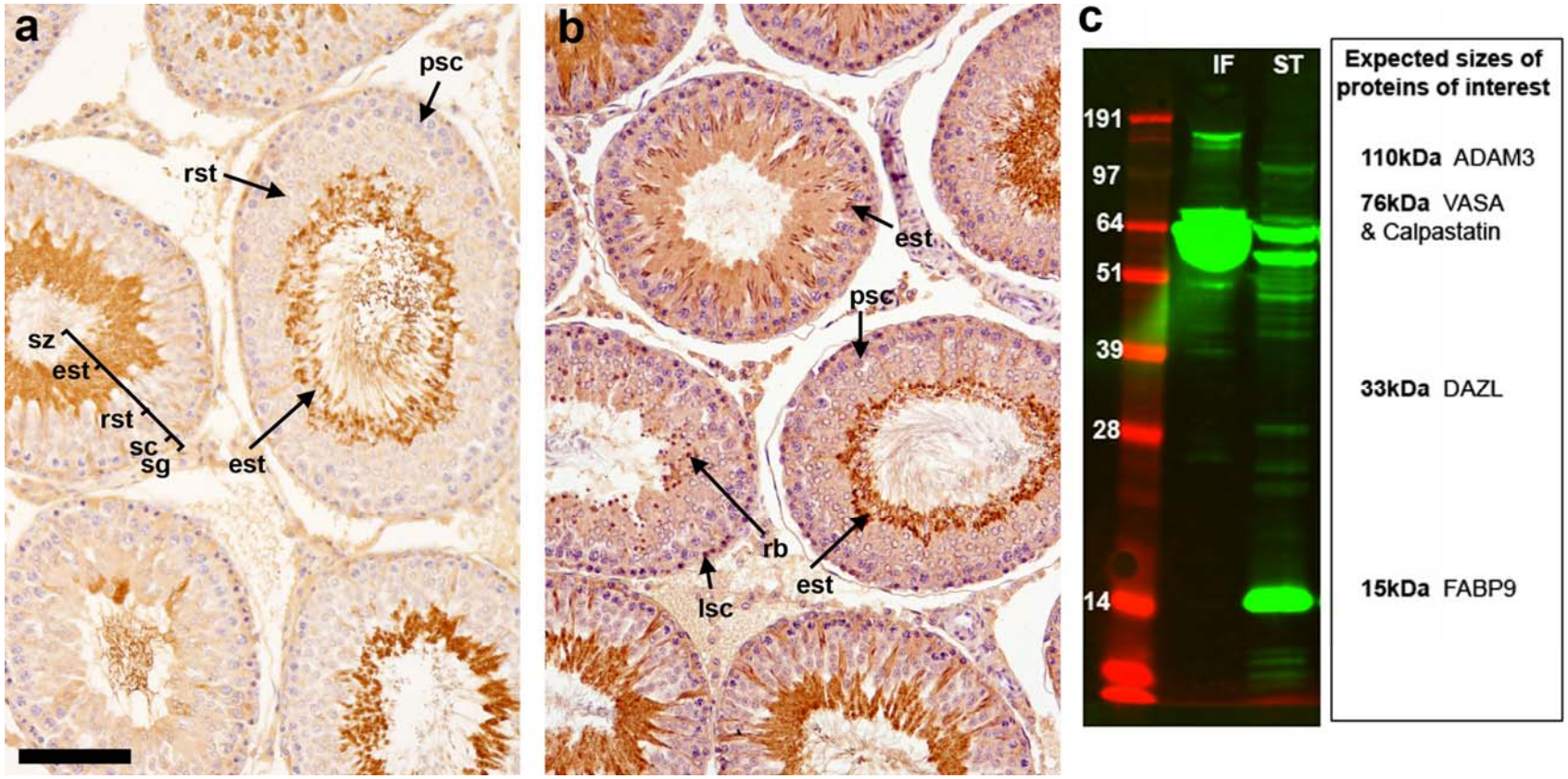


Figure 4-1 Antibody raised to seminiferous tubule conditioned medium. a, b) Immunohistochemistry with antibody to STCM (brown) on control testis sections, counterstained with haematoxylin (blue). A variety of undefined proteins are detected and show stage- and germ cell- dependent expression. sg- spermatogonia, sc- spermatocytes, lsc- leptotene spermatocytes, psc- pachytene spermatocytes, rst- round spermatids, est- elongate spermatids, sz- spermatozoa, rb- residual bodies (please refer to Figure 2-3 for further information about the spermatogenic cycle). Scale bar represents 100μm. c) Panel from Western blot with antibody to STCM and control interstitial fluid (IF) and control seminiferous tubules (ST) to illustrate the variety of proteins the antibody detects. The box indicates expected sizes of specific proteins investigated later in this chapter (molecular weights suggested in antibody manufacturers literature).

Figure 4-1 illustrates the results obtained when the STCM antibody was used for immunohistochemistry on control testis sections (a, b), and Western blotting with interstitial fluid and seminiferous tubule samples from control animals (c). Immunohistochemistry shows the range of proteins detected at different locations in the seminiferous tubules. Stage specific differences in the staining patterns can be seen in both panels a and b, with intense staining in the elongate spermatids and weaker staining in the round spermatids. Panels a and b also illustrate the organisation of spermatogenesis in the seminiferous tubules (refer to Section 2.3.4.4 and Figure 2-3). Western blotting also shows the range of proteins detected by the STCM antibody with many protein bands present in the seminiferous tubule sample (Figure 4-1c), and a smaller number of these proteins detected in the interstitial fluid sample. The sizes of some proteins of interest, discussed later in this chapter and chapter 7 are also indicated in Figure 4-1c to illustrate the range of sizes of proteins investigated and their relation to the protein bands seen with the STCM antibody (the labels do not necessarily mean that the bands observed are the proteins indicated).

4.3.2 Seminiferous Tubule Proteins at Different Stages of Spermatogenesis

As shown in Figure 4-1a and b, different proteins are present in each tubule depending on the stage of spermatogenesis. To investigate whether the stage of spermatogenesis might have an affect on the proteins leaking out of STs into IF, STs were dissected out of untreated testes, and staged using light microscopy. Stages I-V, VI-VIII and IX-XIV can be easily identified depending on the transillumination pattern seen in the tubules (Figure 4-2). Staged tubules were homogenised and run on a Western blot with the STCM antibody (Figure 4-3).

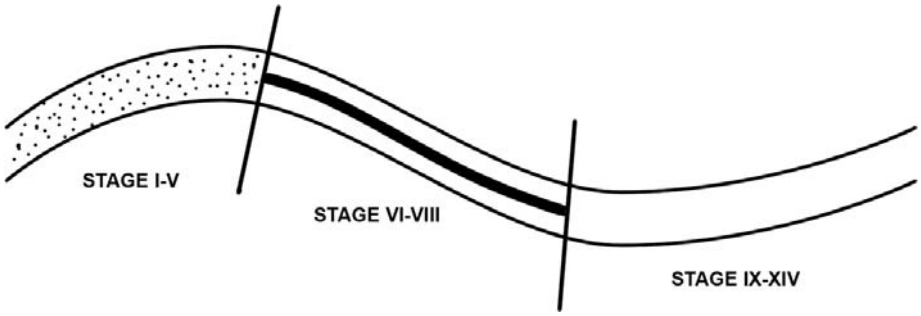


Figure 4-2 Staging Seminiferous Tubules. Tubules were dissected into three stage groups (stages I-V, VI-VIII and IX-XIV) according to the transillumination pattern seen in the tubule.

The Western blot again highlights the range of proteins detected by the STCM antibody. Two separate samples for each group of stages were run on the blot for comparison. The blot also illustrates the variability that naturally occurs between different samples, as well as the variability involved with experimental techniques. For these reasons it is important to run more than one sample from each group so that account can be taken of this variation and the chance of spurious findings minimised.

The Western blot in Figure 4-3a does not appear to show any major differences in the proteins detected between each stage group. A selection of bands were quantified from the Western blot, to allow an objective comparison of the different groups with the mean for each group plotted on graphs (Figure 4-3b). The graphs agreed with the observation that there are no major differences in the intensity of the protein bands across the stage groups, although there was a trend for a greater intensity of protein bands at 51kDa, 39kDa, and 27kDa in size with the VI-VIII staged group compared to the other two staged groups.

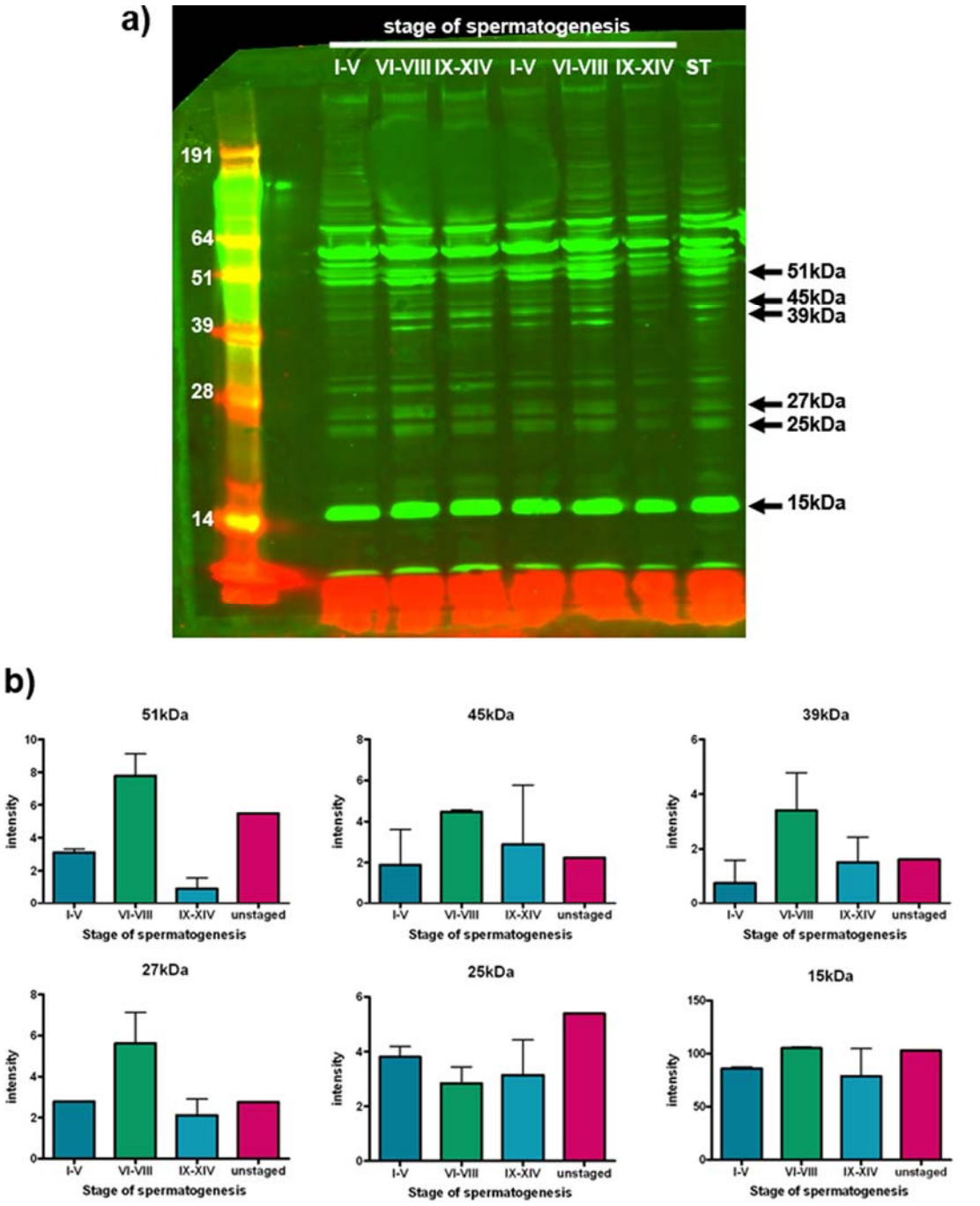


Figure 4-3 a) Western Blot with antibody to STCM, showing staged seminiferous tubules and control unstaged STs. Arrows indicate examples of protein bands quantified. b) Graphs showing quantification of 51kDa, 45kDa, 39kDa, 27kDa, 25kDa and 15kDa bands (mean with SEM) with values taken from samples run on one blot (n=2 samples/staged group).

These graphs illustrate how useful it is to be able to quantify the protein bands and calculate averages to compare across groups. Throughout this thesis, at least two samples for each group have been run on Western blots in order to carry out statistical analyses and comparisons between groups.

In order to further investigate the proteins present in STs, staged STs were also run on a 1D gel and stained with a Coomassie dye based stain to look at total protein content (Figure 4-4). As with the Western blot (Figure 4-3) there do not appear to be any obvious differences in the proteins present in each staged group.

A variety of protein bands were quantified (Figure 4-4b). The graphed results agreed with the observations and suggested there were no differences between the staged groups.

Both Western blots with the general STCM antibody and Coomassie stained gels have been used throughout this thesis for the analysis of IF and ST samples. Both techniques have a role in the identification and comparison of proteins. The STCM antibody detects a range of seminiferous tubule derived proteins and can also be used with immunohistochemistry to look at the localisation of the proteins in testis sections. In comparison, the Coomassie gels detect proteins present in the samples, regardless of origin. However, only 1D gels have been used and so proteins of similar sizes may be masked in one band. Coomassie gels could still be useful for detecting proteins that change across groups but aren't detected with the STCM antibody.

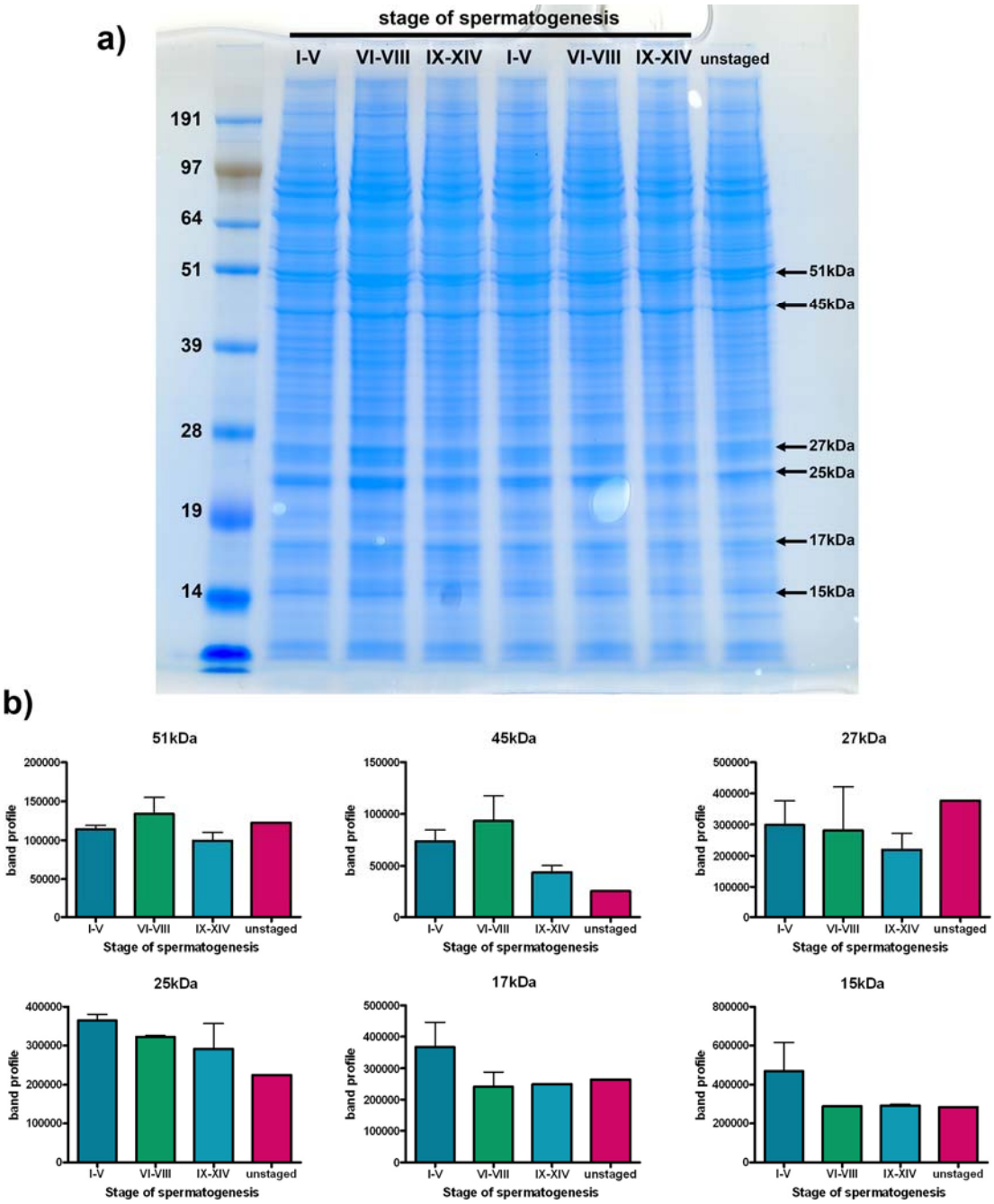


Figure 4-4 Coomassie stained gel showing staged STs and unstaged STs. Arrows indicate examples of protein bands quantified. b) Graphs showing quantification of indicated bands (mean with SEM) with values taken from samples run on one blot (n=2 samples/staged group).

4.3.3 Specific Protein Investigations

The primary aim of this thesis was to evaluate the potential use of biomarkers for testicular toxicity. Therefore, as well as looking generally at the proteins present in control and treated samples, specific candidate biomarker proteins were also investigated. This section of work, including the background to selecting specific proteins, is described thoroughly in chapter 7. Some of these proteins are mentioned in earlier chapters with toxicant treatments, and so it is important to understand how they are expressed in control testicular tissue.

Immunohistochemistry for five germ cell proteins is shown in Figure 4-5. The proteins investigated were:

- A disintegrin and metalloprotease domain3 (ADAM3)
- Calpastatin
- DAZL
- Fatty acid binding protein 9 (FABP9)
- VASA

The immunohistochemical staining clearly shows the localisation of each protein, and stage specific expression. ADAM3 was expressed in round and elongate spermatids (Figure 4-5a). Staining for calpastatin was also in round spermatids (Figure 4-5b). DAZL was localised to pachytene spermatocytes and residual bodies towards the lumen of the tubules (Figure 4-5c) and VASA was localised to both spermatocytes and spermatids (Figure 4-5e). Staining for FABP9 was not as distinct as the four other proteins, but suggested localisation of FABP9 to spermatocytes and spermatids (Figure 4-5d).

As well as immunohistochemical analysis to investigate the localisation of the proteins in testicular sections, analysis of the specific proteins in interstitial fluid and seminiferous tubule samples was carried out using Western blotting with the specific antibodies to the proteins. An example of a Western blot for FABP9 with seminiferous tubules is shown in Figure 4-5f. One clear band can be seen in the

sample, about 15kDa in size (the expected size for FABP9), showing the specificity of the antibody to detect the protein in seminiferous tubule samples. The expected molecular weights of the five proteins of interest are shown in Figure 4-1c.

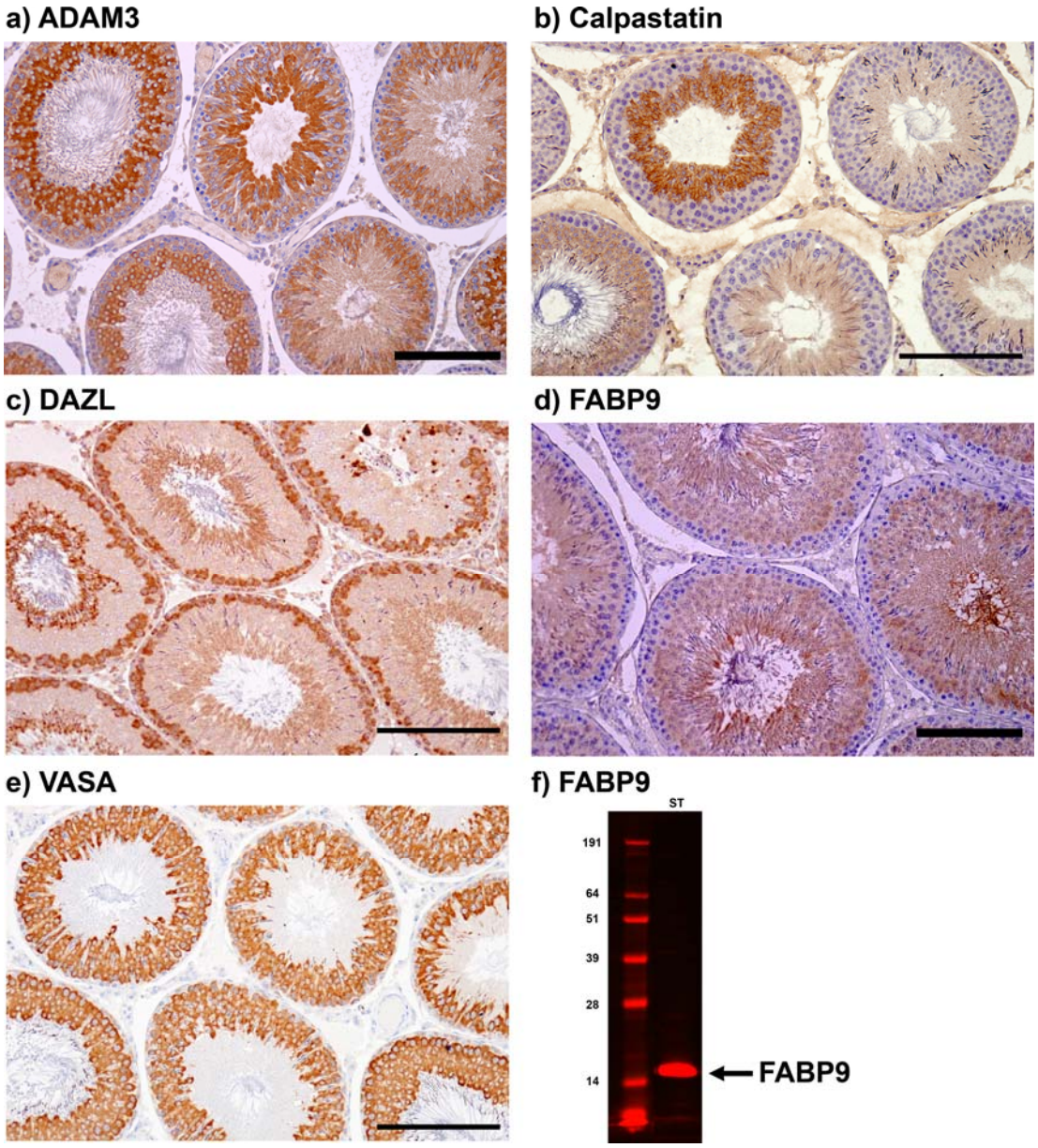


Figure 4-5 Testis sections from control adult rats, immunostained (brown) to show a) ADAM3, b) Calpastatin, c) DAZL, d) FABP9 and e) VASA, all counterstained with haematoxylin (blue). Scale bars represent 100µm. f) Panel from Western blot with FABP9 and seminiferous tubule extract (ST).The FABP9 band is indicated by an arrow (15kDa).

4.3.4 Blood-Testis Barrier Investigations

In order to investigate the hypothesis that proteins may leak from seminiferous tubules into interstitial fluid due to loss of integrity of the blood-testis barrier (BTB) or germ cell damage, the integrity of the BTB was evaluated following toxicant treatments.

This section serves to show the presence of the BTB in control testis sections, an appreciation of which is necessary before the state of the barrier following toxicant treatment can be evaluated.

The BTB is made up of a range of different interacting proteins forming tight junctions and adherens junctions (described in section 2.3.6). Studies published in the literature regularly use immunofluorescent techniques to evaluate the integrity of the BTB in testis sections. Following on from this, double immunofluorescence co-staining with tight junction proteins, occludin and ZO-1, and claudin-11 and ZO-1, and adherens junction proteins, N-cadherin and β-catenin, were carried out to evaluate the BTB in the toxicant studies. These proteins were chosen as they have been widely used in the literature (Wong et al., 2004; Wong et al., 2005; Yan and Cheng, 2005; Li et al., 2006; Xia et al., 2006; Wong et al., 2007; Kopera et al., 2009) and provide combinations of junction and accessory proteins, which should co-localise at the site of the BTB.

A second immunofluorescent technique for evaluating the integrity of the BTB, using fluorescent detection of a biotin tracer injected into the testes, was also carried out (sections 3.3.6 and 3.6.7). The site of the BTB was illustrated in these studies by immunofluorescent detection of occludin.

4.3.4.1 Tight Junctions

The blood-testis barrier forms between post-natal days 15-19 in the rat (Vitale et al., 1973; Russell et al., 1989). Figure 4-6 illustrates the presence of both ZO-1 and

occludin at the site of the BTB in day 25 tubules, whereas at day 15, only ZO-1 the adaptor protein can be seen and it is not yet localised to the site of the blood-testis barrier.

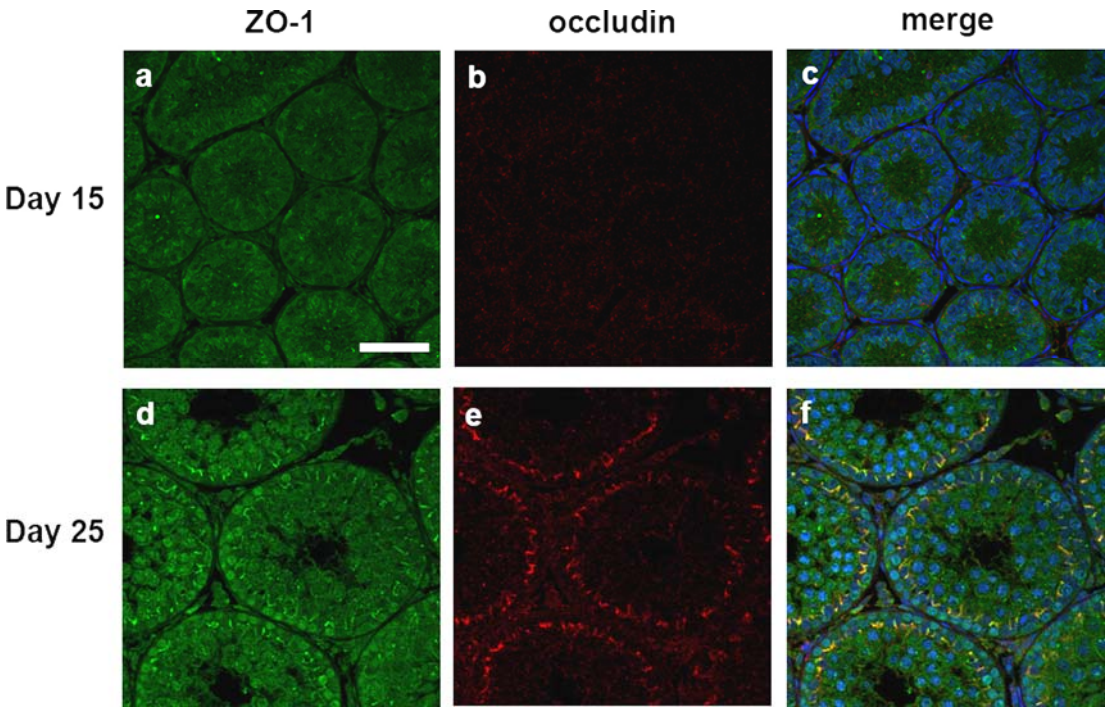


Figure 4-6 Immunofluorescence staining for a, d) ZO-1 (green), b, e) occludin (red) and c, f) merged image with Dapi nuclear counterstain (blue), with testis sections from rats aged 15 (a, b, c) or 25 (d, e, f) days. Scale bar represents 50μm.

Evaluation of the integrity of the tight junctions in the blood-testis barrier is widely carried out by looking at the co-localisation of a junction and an adaptor protein. Two different co-localisation studies have been used to investigate the tight junctions in the BTB in this thesis; occludin and ZO-1, and claudin-11 and ZO-1. Figure 4-7 shows immunofluorescent co-localisation of these two protein combinations in control tissue sections.

Occludin and ZO-1 clearly co-localise at the site of the BTB in tubules shown in Figure 4-7a-c. Unfortunately there is a lot of red background in these pictures; this is due to non-specific binding of the tyramide (used in the detection of occludin) to the interstitum. Figure 4-7d-f shows a similar pattern, although the absence of occludin

from one seminiferous tubule can be noted. As described in chapter 2, a gross restructuring of the BTB during stages VII-IX must take place to allow the spermatocytes to pass through the barrier and continue their developmental path moving towards the lumen of the tubule. This restructuring is thought to involve an engagement/disengagement mechanism with the adherens and tight junction protein complexes (described in section 2.3.6.4, Yan and Cheng, 2005). It has been reported in one study that occludin cannot be identified in stage VIII tubules (Li et al., 2006). Despite co-localisation of occludin and ZO-1 being one of the most widely used ways of showing the presence of the BTB, this is the only reference to the absence of occludin at stage VIII. This observation does, however, fit with the proposed mechanisms of BTB restructuring at stages VII-IX. The tubule in Figure 4-7f appears to be at stage VIII, so this could explain why occludin is not observed in this tubule. The stage of the tubule may, therefore be an important consideration when evaluating the integrity of the BTB through co-localisation of occludin and ZO-1.

Localisation of a further tight junction protein, claudin-11, which also has ZO-1 as an adaptor protein (section 2.3.6.1), was also assessed to ensure results obtained with occludin/ZO-1 provide a true representation of damage to the BTB and not just to occludin in the tight junctions. Figure 4-7 panels g-i show the co-localisation of claudin-11 and ZO-1 at the site of the BTB. When looking at these figures, it is important to remember that the BTB is a 3D structure. The position of the proteins clearly changes as you adjust the z-plane of the image taken through the slide, showing how the proteins move up and down linking the Sertoli cells. The best possible picture to show the localisation of both proteins of interest was taken.

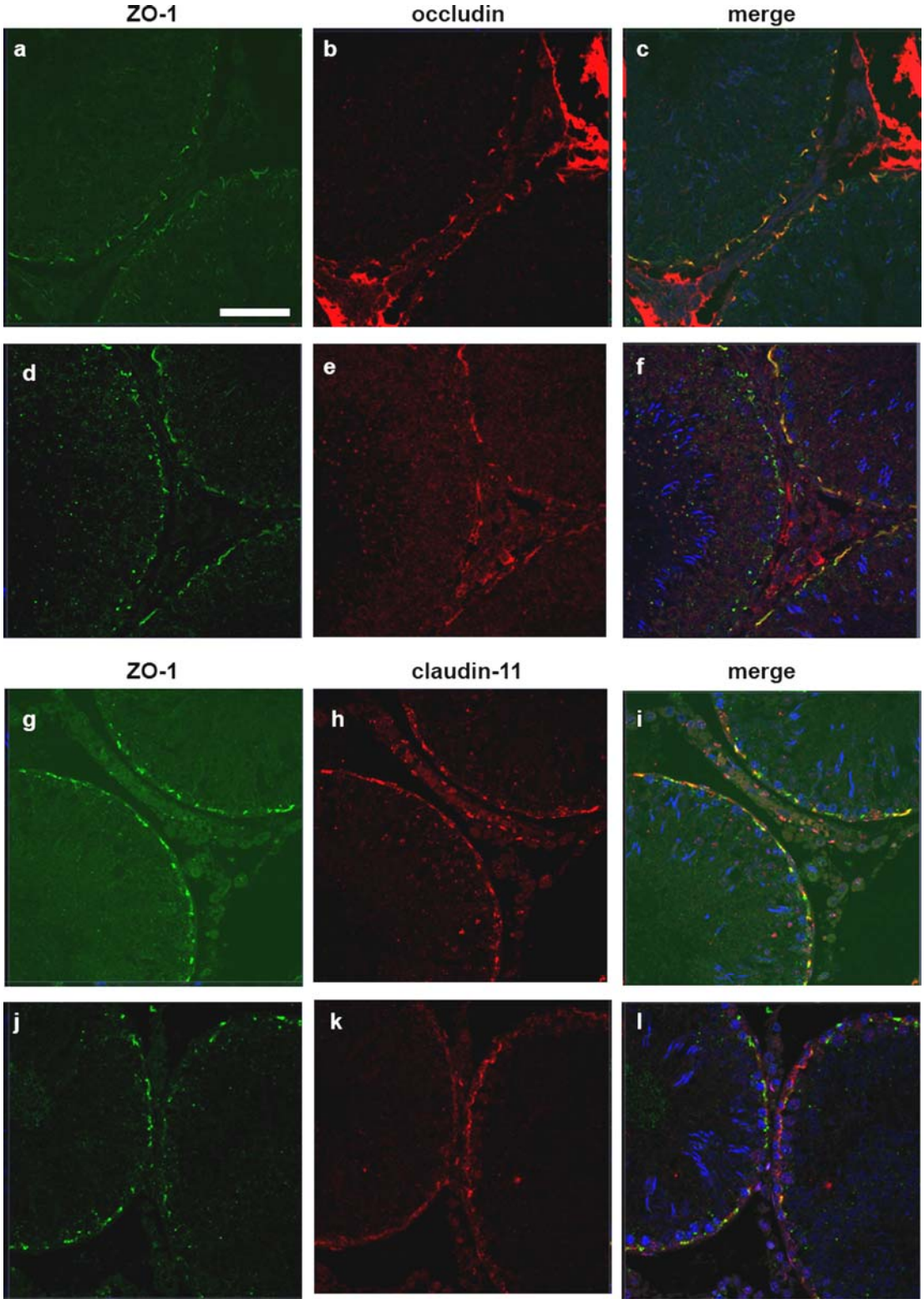


Figure 4-7 Immunofluorescence staining for a, d) ZO-1 (green), b, e) occludin (red) and c, f) ZO-1/occludin merged image, g, j) ZO-1 (green), h, k) claudin-11 (red), i. l) ZO-1/claudin-11 merged image with Dapi nuclear counterstain (blue), on testis sections from control adult rats. Scale bar represents 50μm.

The tubules in Figure 4-7 panels j-l, do not show co-localisation of claudin-11 and ZO-1 as clearly as panels g-i. This illustrates the variability of the results obtained. Particularly with this double immunofluorescent protocol, staining was very patchy in some sections, making it hard to evaluate the junction proteins. The tubule on the right hand side of the figure (panels j-l) shows the presence of both claudin-11 and ZO-1, although little co-localisation. This could be due to the 3D nature of the structure, and ZO-1 could be localised just below the claudin-11 in the z-plane. It could also be due to the problems of patchy staining observed with this protocol. The second tubule on the left hand side (panels j-l) appears to show the absence of claudin-11 at the site of the BTB. Again this could be due to the method difficulties. Alternatively, it could be due to the same reason as for the absence of occludin observed in the tubule in panels d-f. This tubule also appears to be stage VIII, and claudin-11 could be reduced/absent from the BTB during restructuring in the same way that occludin is.

4.3.4.2 Adherens Junctions

As described in chapter 2 (section 2.3.6.2), adherens junctions are another component of the BTB. Co-localisation of an adherens junction protein, N-cadherin, and an adaptor protein, β-catenin, was investigated to allow an evaluation of other factors affecting the BTB, rather than just an evaluation of the tight junctions.

Figure 4-8 shows the natural localisation of these two proteins in seminiferous tubules. N-cadherin can be clearly seen at the site of the BTB (Figure 4-8c). As well as forming Sertoli cell-Sertoli cell junctions, adherens junction proteins can also form Sertoli cell-germ cell connections. In panels c and d, the N-cadherin can be seen extending around the germ cells as well as the clear line of the BTB. The localisation of β-catenin can also be clearly seen in panels b and d. β-catenin appears to be localised to the nuclei of the germ cells. Although these two proteins do not co-localise, they are both involved in formation of adherens junctions, and their localisation provides a further evaluation of the integrity of the BTB.

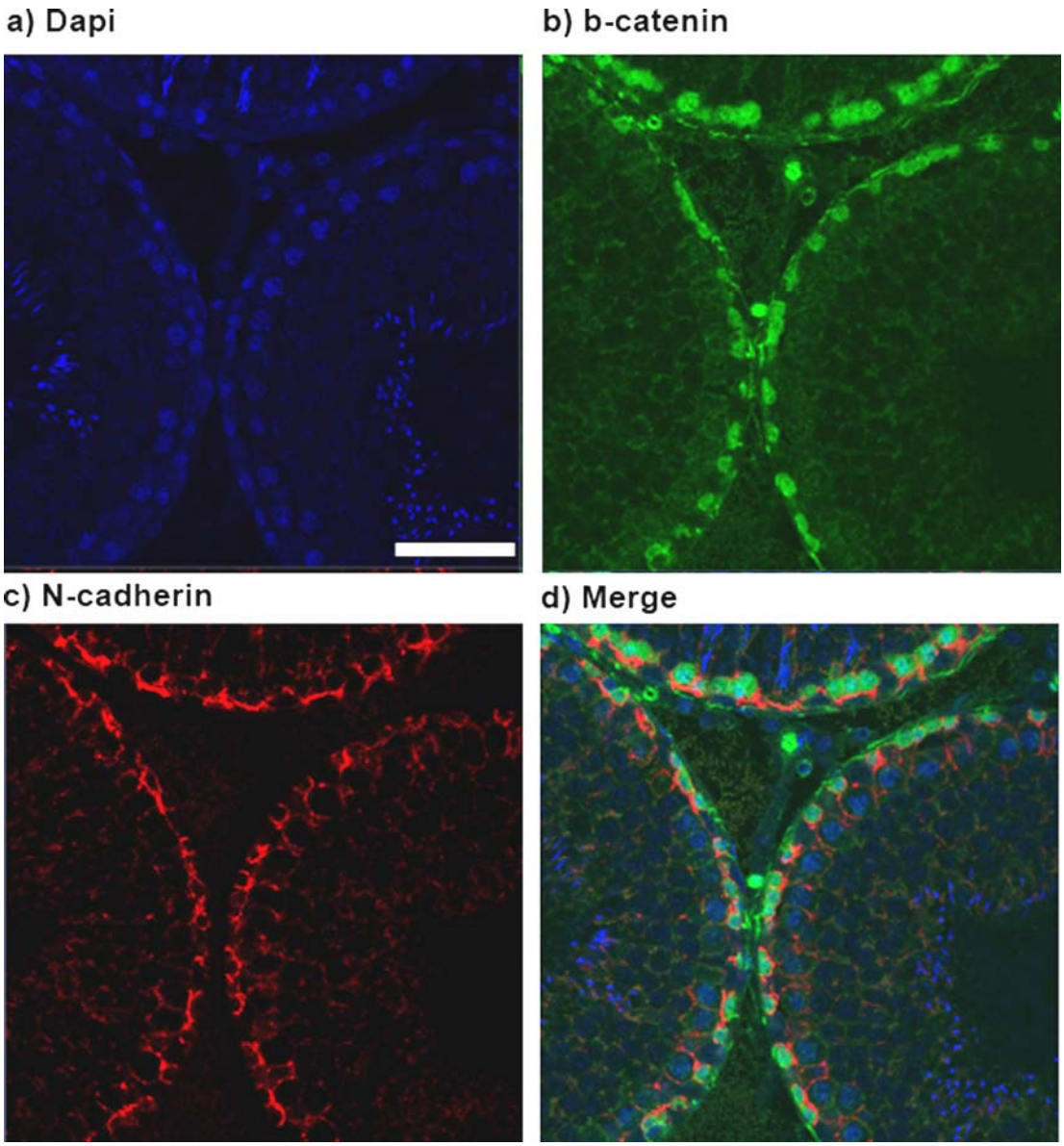


Figure 4-8 Immunofluorescence staining for a) Dapi counterstain (blue), b) β-catenin (green), c) N-cadherin (red) and d) β-catenin/N-cadherin merged image on a testis section from a control adult rat. Scale bar represents 50μm.

4.3.4.3 Biotin Tracer

The second technique used for evaluation of the integrity of the BTB involved fluorescent detection of a biotin tracer that was injected into the testes before fixation. This method provides a technique for evaluation of the function of the BTB, which complements evaluation of the integrity of the structure of the BTB

described above. The site of the BTB was also illustrated in these studies by immunofluorescent detection of occludin. The biotin tracer (EZ-link sulfo-NHS-LC-biotin (Pierce Bioscience, IL, USA)) is an ester of biotin, which is water soluble, but cannot penetrate cell membranes due to a charged sulphonate group. If the BTB is functional, the biotin tracer should be detected in the interstitum and seminiferous tubules up to the site of the BTB. If the functional integrity of the BTB has been lost, the biotin tracer should penetrate the barrier and be detected inside of the tubules (Tarulli et al., 2008).

Figure 4-9 shows images from control testes, which were injected with the biotin tracer. These panels illustrate the variation that can be seen with this technique in control tissue, supporting the need for large n numbers, and analysis of control tissue. Panel a clearly shows that the biotin tracer is restricted to the interstitum. Unfortunately the tyramide detection system used for detection of occludin, also binds to non-specific proteins in the interstitum, creating red background and turning the green detection of the biotin tracer yellow (panel a). Panels c and d also show a clear distribution of the biotin tracer in the interstitum. It can also be seen surrounding germ cells on the interstitial side of the BTB, particularly in panel c.

Figure 4-9b, f and g also show the biotin tracer in the interstitum. However, in these pictures, the tracer appears to have penetrated into the tubules, even though the BTB is still intact (shown by the presence of occludin). The pattern observed suggested that the tracer is moving into the tubules between Sertoli cells. The pattern does not suggest total loss of integrity of the BTB, and is perhaps caused by looking at an area of the testis close to the injection site, so a larger volume of tracer was present, or due to leaving the testis incubating with the injected tracer for slightly longer than other testes. Alternatively, this observation could be due to hydrolysis of the biotin tracer. Although the tracer solution was freshly made before sample collection, it is possible that the hydrolysis of the tracer occurred before it was injected into some of the tissue samples collected later during the collection period, and this could have affected the permeability of the tracer. Again, this illustrates the importance of

evaluating the variation in control tissue before drawing conclusions with results from treated animals.

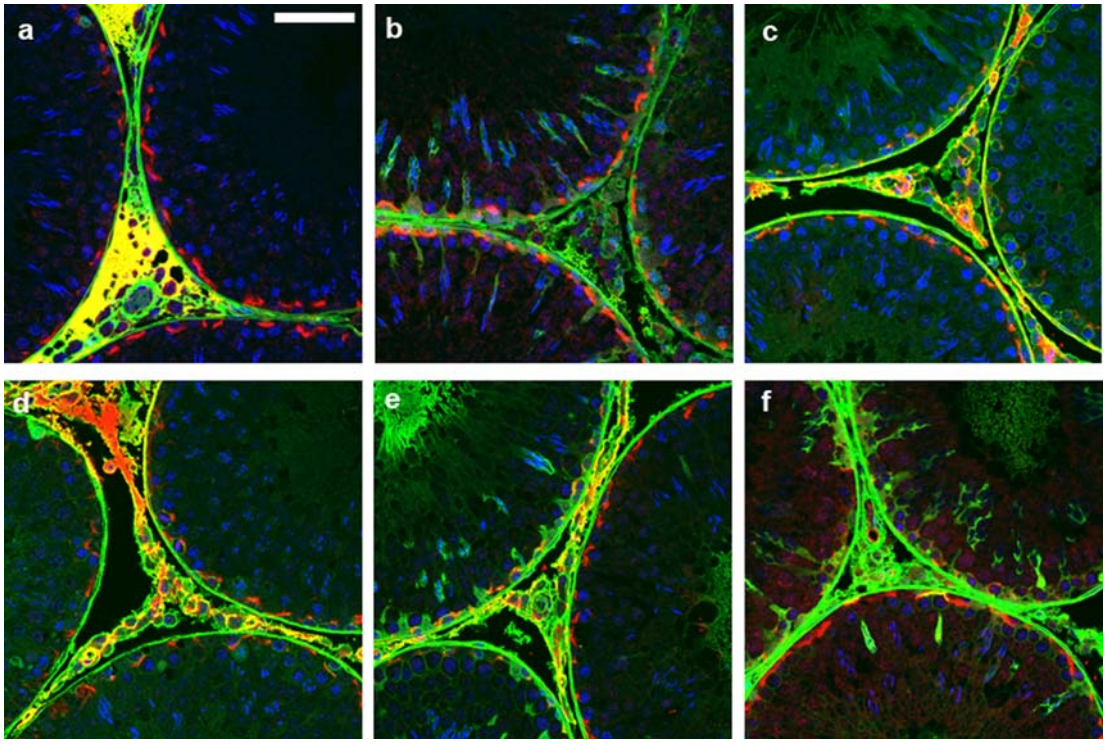


Figure 4-9 Detection of biotin tracer (green) and occludin (red) with Dapi nuclear counterstain, on testis sections from control adult rats. Scale bar represents 50µm.

4.4 Discussion

The aim of this chapter was to introduce the techniques, tools and approaches used to investigate the potential use of biomarkers for testicular toxicity, as well as providing a reference for the natural variation seen with the experimental methods in testes from control rats. It is important to understand this variation before an objective evaluation of tissue from treated animals can be carried out.

Three approaches have been used to investigate potential biomarkers and the hypothesis that germ cell derived proteins may leak from seminiferous tubules into interstitial fluid due to loss of integrity of the blood-testis barrier or as a direct result of germ cell damage following toxicological insult to the testis. A general antibody

to seminiferous tubule derived proteins (STCM antibody) was used to evaluate changes in a range of seminiferous tubule derived proteins. 1D gels stained with a Coomassie dye based stain were used to assess total protein content in samples, and specific biomarker candidate proteins were also investigated.

An antibody raised to seminiferous tubule conditioned medium (STCM) was prepared by a previous PhD student (Katie Turner, Turner, 1996), and used to investigate changes in proteins in samples from heat treated testes in her studies. The antibody has been used in these studies in a similar way to investigate differences in proteins present in samples from control and toxicant-treated animals. As shown in Figure 4-1, the antibody was used to detect a range of proteins in testis sections by immunohistochemistry, with stage specific staining, and intense staining in the elongate spermatids and weaker staining in the round spermatids. Although the proteins are undefined, immunohistochemistry with the antibody to STCM can be used to obtain an idea of any changes to the seminiferous tubules following toxicant treatment. Western blotting with a homogenised seminiferous tubule sample and interstitial fluid sample also showed a range of proteins of different sizes. Western blotting with the STCM antibody can be used in investigations into leakage of proteins from STs into IF following toxicant treatment by providing an assessment of a range of seminiferous tubule derived proteins in interstitial fluid samples.

Immunohistochemistry with the antibody to STCM showed that different proteins are present in STs at different stages of spermatogenesis. The stage of spermatogenesis might have an affect on the proteins leaking out of STs into IF, so this was investigated by dissecting seminiferous tubules from untreated testes, and staging them into three groups (I-V, VI-VIII, IX-XIV) using transillumination. Homogenised staged tubules were run on a Western blot with the antibody to STCM to compare the proteins present. Some sample variability was observed which could be due to natural variability, but no obvious differences in the proteins were observed between the different staged groups. Quantification of some of the proteins bands did, however, suggest an increase in the concentration of three proteins (51kDa, 39kDa

and 27kDa) with the VI-VIII group. These results showed that it is essential to run at least two samples/group on Western blots to allow for sample variability and also that it is useful to quantify bands on Western blots and calculate averages across different groups

The differences in proteins observed with the staged STs and Western blot with the STCM antibody were further investigated by running the samples on a 1D gel and staining with a Coomassie dye based stain to assess the total protein content of the samples. Many more protein bands were observed than with the STCM antibody, and quantification of a range of proteins suggested that there are no differences in the concentrations of the proteins across the different staged groups.

The staged ST investigations introduced the techniques used to evaluate the proteins in IF and ST samples in the toxicant studies. Western blotting with the STCM antibody has a role in evaluating a range of different ST-derived proteins, and can also be used for immunohistochemistry to look at the localisation of the proteins in testis sections. Coomassie stained gels have a complementary role, in that they can be used to detect proteins in the sample, regardless of origin. However, it is important to note, that only 1D gels have been used, and so proteins of similar sizes may be masked in one band. Coomassie gels could still be useful for detecting proteins that change across groups but aren't detected with the STCM antibody in the evaluation of leakage of proteins from STs into IF.

The third approach taken to investigate the potential for biomarkers for testicular toxicity was the selection of potential biomarker candidate proteins, from an Affymetrix Genechip® Screen carried out at GSK. The specific protein investigations and reasons for selection of the proteins are discussed in chapter 7, but the techniques for investigating them are introduced here. Specific antibodies to the proteins of interest (ADAM3, calpastatin, DAZL, FABP9, and VASA) were used with immunohistochemistry to assess the localisation of the proteins, and later on to evaluate any changes following toxicant treatment. The results presented in Figure

4-5 show the specificity of the antibodies when used for immunohistochemistry with testis sections from control rats. The localisation and stage-specificity of each protein can be identified, although the staining for FABP9 was not as distinct as the other four proteins. The specific antibodies were also used for Western blotting with IF and ST samples. Figure 4-5f shows an example of a blot with the FABP9 antibody showing the detection of the protein in a ST sample by the presence of one clear band at the expected size of the FABP9 protein (15kDa).

Finally, in order to investigate the hypothesis that proteins may leak from seminiferous tubules into interstitial fluid following toxicological damage, and whether such leakage is due to loss of integrity of the blood-testis barrier or direct germ cell damage, analysis of the blood-testis barrier must be carried out. This chapter introduced the techniques used for BTB evaluation in later chapters. Two methods were used, immunofluorescent co-staining of blood-testis barrier proteins (junction protein and adaptor protein), and immunofluorescent detection of a biotin tracer, injected into freshly dissected testes as an evaluation of BTB function.

The blood-testis barrier forms between days 15-19 in the rat (Vitale et al., 1973; Russell et al., 1989). Figure 4-6 supports the literature, and showed the presence of the tight junction proteins, occludin and ZO-1 present and co-localised at the site of the BTB in day 25 rats but absent in day 15 rats. Further investigations involving development of the BTB are discussed in chapter 6.

To evaluate the BTB in adult testes, immunofluorescent co-staining was carried out for the tight junction pairs, occludin/ZO-1, and claudin-11/ZO-1, and the adherens junction pair, N-cadherin/β-catenin, to obtain an assessment of different structural components of the BTB. Occludin and ZO-1 are widely used to illustrate the integrity of the BTB in published studies (Wong et al., 2004; Wong et al., 2005; Yan and Cheng, 2005; Li et al., 2006; Xia et al., 2006; Wong et al., 2007; Kopera et al., 2009). Clear and consistent co-localisation of occludin and ZO-1 was observed in control testis samples, although the absence of occludin was noted in some

seminiferous tubules. These tubules were noted to be at stage VIII of spermatogenesis, the stage at which the preleptotene spermatocytes traverse the BTB. This observation has been described in the literature (Li et al., 2006), although only in one study, which is surprising considering how often occludin is used as a marker for the BTB. Co-staining for claudin-11 and ZO-1 was less consistent than with occludin/ZO-1, however, both proteins could be seen to be localised to the site of the BTB in some tubules. On the other hand, some tubules showed only expression of one of the proteins. This could be due to the 3D nature of the structure of the BTB, with one protein localised below the other in the z-plane. It could also be due to the problems with the staining protocol, or alternatively a stage specific effect, as seen with occludin. Due to the more consistent immunofluorescent staining with occludin/ZO-1, this protocol was used in all investigations to evaluate the tight junctions in the BTB.

The adherens junctions were assessed by co-staining for N-cadherin and β-catenin. N-cadherin was localised to the site of the blood-testis barrier as well as extending around the germ cells. Adherens junction proteins form interactions between Sertoli-Sertoli cells and Sertoli-germ cells explaining this observation. Co-localisation of N-cadherin and β-catenin was not observed. Instead β-catenin was localised to the nuclei of the preleptotene/leptotene spermatocytes, either side of the blood-testis barrier. Although the proteins do not co-localise, they are both found in the same area of the tubules, and could be interacting via other adaptor proteins. Co-staining of N-cadherin and β-catenin is still a useful tool in the assessment of the adherens junctions in the blood-testis barrier.

The second technique used for evaluation of blood-testis barrier function was the biotin tracer technique. The biotin tracer, an ester of biotin that cannot penetrate cell membranes, was injected into freshly dissected testes, and then detected in the fixed tissue by immunofluorescence. If the BTB is functional, then the tracer should localise in the interstitum and only in the seminiferous tubules up to the site of the BTB. This localisation of the tracer was observed in most control seminiferous

tubules. There was, however, some variation in the localisation of the tracer in some tubules with some detection of biotin in areas of the adluminal compartment, although complete penetration of the tracer into the tubule was not observed in any control tissue. The staining patterns observed do not suggest a complete loss of integrity of the blood-testis barrier, although could be due to the tracer passing through the barrier during restructuring of the junction proteins. It could also be an effect of the experimental technique and found in areas of the testis close to the injection site, so a larger volume of tracer was present, or due to leaving the testis incubating with the injected tracer for slightly longer than other testes, or potentially due to hydrolysis of the biotin tracer before it was injected. Although some variability was observed with this technique, with analysis of control tissue samples, and large n numbers, reliable results were still obtained.

The results presented in this chapter have introduced the concepts and techniques used in this investigation of the potential use of biomarkers for testicular damage, as well as providing a forewarning of the variation observed in control testicular tissue samples in the experimental approaches used in this thesis.

5 Leakage of Proteins from Seminiferous Tubules following the Administration of known Testicular Toxicants

5.1 Introduction

Following disruption of spermatogenesis, it is thought that germ cell proteins may leak from seminiferous tubules (STs) in the testes into interstitial fluid (IF) (Turner et al., 1996). These proteins have the potential to then diffuse into the bloodstream in the testis (Figure 5-1). A highly sensitive detection system could then be used to detect changes in the level of the protein/proteins, and provide a biomarker for testicular toxicity.

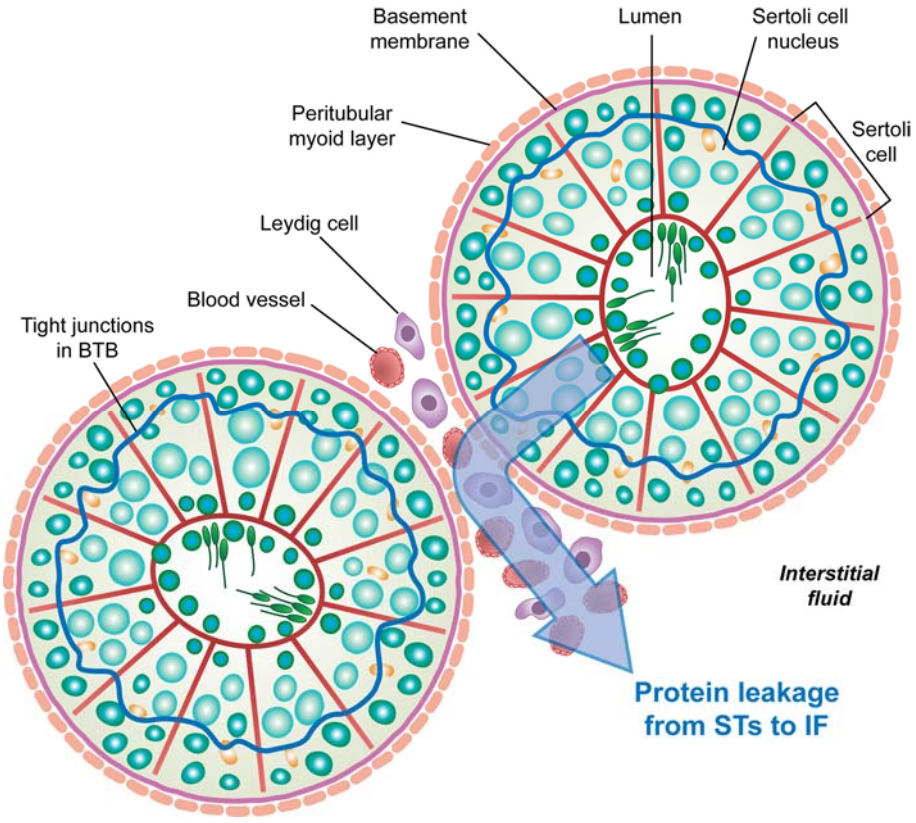


Figure 5-1 Germ cell derived proteins may leak from seminiferous tubules into interstitial fluid following disruption of spermatogenesis.

In order to investigate this hypothesis, it must first be established whether proteins do leak from seminiferous tubules into interstitial fluid following toxicological damage, and what the conditions leading to such leakage might be. Protein leakage from STs into IF could be caused by either germ cell damage or loss of integrity of the blood-testis barrier. To investigate this, three model testicular toxicants were chosen; cadmium chloride, methoxyacetic acid and 1,3-dinitrobenzene, which specifically target different components of seminiferous tubules.

5.1.1 Cadmium Chloride

Cadmium chloride ($CdCl_2$) is a well used tool for both in vivo and in vitro investigations involving the blood-testis barrier. It has been shown to cause disruption of tight junctions in vitro (Janecki et al., 1992) and in vivo (Hew et al., 1993b), and has been shown to cause a stage-specific failure of spermiation (Hew et al., 1993a). However, the mechanism of action of cadmium chloride on the testes is not fully understood. Cadmium toxicity to humans is of increasing concern, and so more and more studies investigating the mechanism of such toxicity are emerging. A review of the current ideas about the mechanism of cadmium toxicity can be found in chapter 2.

The widely studied nature of the effects of cadmium chloride makes it an ideal model toxicant to investigate disruption to the blood-testis barrier, and whether this leads to leakage of proteins from STs into IF.

5.1.2 Methoxyacetic Acid

Methoxyacetic acid (MAA) is another well-studied testicular toxicant. It has a stage-specific effect causing the depletion of pachytene spermatocytes (Bartlett et al., 1988). The damage is thought to be caused by a disruption of a signal required to maintain cell viability, because spermatocytes appear to undergo apoptosis (Bagchi and Waxman, 2008). The specific target of MAA makes it ideal for this study, to

investigate whether damage to the germ cells (specifically pachytene spermatocytes) causes proteins to leak from STs into IF.

5.1.3 1,3-dinitrobenzene

1,3-dinitrobenzene (DNB) or meta-dinitrobenzene is the third well-studied testicular toxicant used in these studies. It has been shown to have effects on Sertoli cells, causing vacuolation and retraction of Sertoli cell cytoplasm in the region of the primary spermatocytes (Blackburn et al., 1988), and also to have effects on spermatocytes and depletion of round spermatids, following prolonged dosing periods. The effects on spermatocytes are thought to be secondary to the Sertoli cell effects (Blackburn et al., 1988), and result in germ cell apoptosis (Muguruma et al., 2005). The target specificity of DNB, and its well studied nature, makes it a useful toxicant for this study, to investigate whether damage to the Sertoli cells causes proteins to leak from STs into IF.

5.1.4 General Aims

The aim of this chapter was to use the three well defined testicular toxicants as tools to investigate whether proteins do leak from the seminiferous tubules into interstitial fluid following toxicological insult to the testes, and whether this leakage is due to loss of integrity of the blood-testis barrier or damage to germ cells.

The approach to investigating this hypothesis involved treatment of adult male rats with the different testicular toxicants, followed by an evaluation of the damage caused using immunohistochemistry, an investigation into the leakage of proteins from STs into IF by Western blot and Coomassie gel analyses, and finally an assessment of the integrity of the blood-testis barrier, using immunofluorescent techniques.

5.2 Methods

5.2.1 Treatments & Sample Numbers

Cadmium chloride, methoxyacetic acid, and 1,3-dinitrobenzene treatments were carried out as described in section 3.3. Tissue was collected for fixation (with and without biotin tracer) for immunohistochemical and immunofluorescent investigations, frozen tissue was collected for PCR analysis, and IF samples were collected for Western blot and Coomassie gel analyses.

Initial studies with cadmium chloride treatment examined the use of three doses, 0.2mg/kg, 1mg/kg, and 3mg/kg cadmium chloride, selected from published literature to show a dose response effect (Hew et al., 1993b; Wong et al., 2004). Evaluation of the samples collected showed that 3mg/kg $CdCl_2$ had a marked effect on the testes, but 0.2 and 1mg/kg did not appear to have any effect. A fourth dose, 1.75mg/kg, was chosen as an intermediate dose for future studies, in place of the 0.2mg/kg dose. Following selection and trials of these doses, two further studies were set up to collect the required tissue. The number of samples collected is shown in Table 5-1. There is some variation in the number of each type of sample/group because extra control fixed tissue from initial studies has been included, and some interstitial fluid samples had dried up in the freezer, due to the small volume collected, before gels and Western blots could be run.

With the MAA studies, an initial investigation showed that 200mg/kg was not a high enough dose to cause significant damage to spermatocytes, so a second dose of 650mg/kg was tested. Varying numbers of animals were used in these studies. A second repeat study was set up to include the biotin tracer analysis and collect further IF samples and frozen tissue. Six animals were used per dose group, collecting 4 testes for each type of sample/group (Table 5-1).

DNB doses, 25 and 50mg/kg, were selected based on previous studies in the laboratory, and published data (Blackburn et al., 1988), as doses expected to cause

mild and moderate effects, respectively. Two studies were run with 4 animals/group in the first study and 6 animals per dose group in the second, collecting 4 testes for each type of sample/group in the second study (Table 5-1).

Table 5-1 Number of Samples collected from $CdCl_2$, MAA and DNB studies. For fixed tissue (n) indicates number of fixed tissue samples injected with biotin tracer.

Toxicant	Dose (mg/kg)	Number of Samples		
		Fixed Tissue	Frozen Tissue	IF
Cd	0	10 (4)	4	7
	1	7 (4)	4	5
	1.75	8 (4)	4	8
	3	7 (4)	4	8
MAA	0	7 (4)	4	10
	200	8 (4)	4	8
	650	6 (4)	4	8
DNB	0	8 (4)	4	8
	25	8 (4)	4	8
	50	8 (3)	4	8

5.2.2 Treatment Effects – Observations

Throughout sample collection during all studies, any differences observed between the dose groups (reaction to treatment, appearance of tissue) were noted. Differences were observed with the higher doses of cadmium chloride (1.75mg/kg and 3mg/kg) and the high dose of DNB (50mg/kg). Table 5-2 shows brief descriptions of the observed differences. It should be noted that the extreme effects seen with 50mg/kg DNB were only observed in the second, repeat, study, and a dose this high would not be used in any future DNB studies. It should also be noted that not all animals in each group exhibited the same observations, for example, testes from one animal in the 3mg/kg $CdCl_2$ group looked completely normal.

Table 5-2 Observations during treatment administration and sample collection for all toxicant studies.

Toxicant	Dose (mg/kg)	Observations
Cd	0, 1	none
	1.75, 3	Animals appeared unwell immediately after dosing and were quiet and sedate. 24hrs later, animals appeared normal, testes purple/dark red appearance, testes more compact/solid but more IF/blood than control
MAA	0, 200, 650	none
DNB	0, 25	none
	50	24hrs after administration, one animal found dead, others looked unwell, with blueness of the extremities (cyanosis)

5.2.3 Treatment Effects – Testis Weight and Interstitial Fluid Volume

As described in sections 3.2 and 3.4.3, testis and interstitial fluid weights were recorded during IF collection. Bodyweights of all animals were also recorded at necropsy. Figure 5-2 shows graphs of average testis weight, and IF weight (expressed as a ratio of testis weight) for each dose group of each treatment. There was a slight trend for an increase in testis weight following cadmium chloride treatment and a statistically significant increase in IF weight following treatment with 1.75mg/kg and a trend for more IF with 3mg/kg $CdCl_2$. No statistically significant effects on testis weight or IF weight were seen with any dose of MAA or DNB.

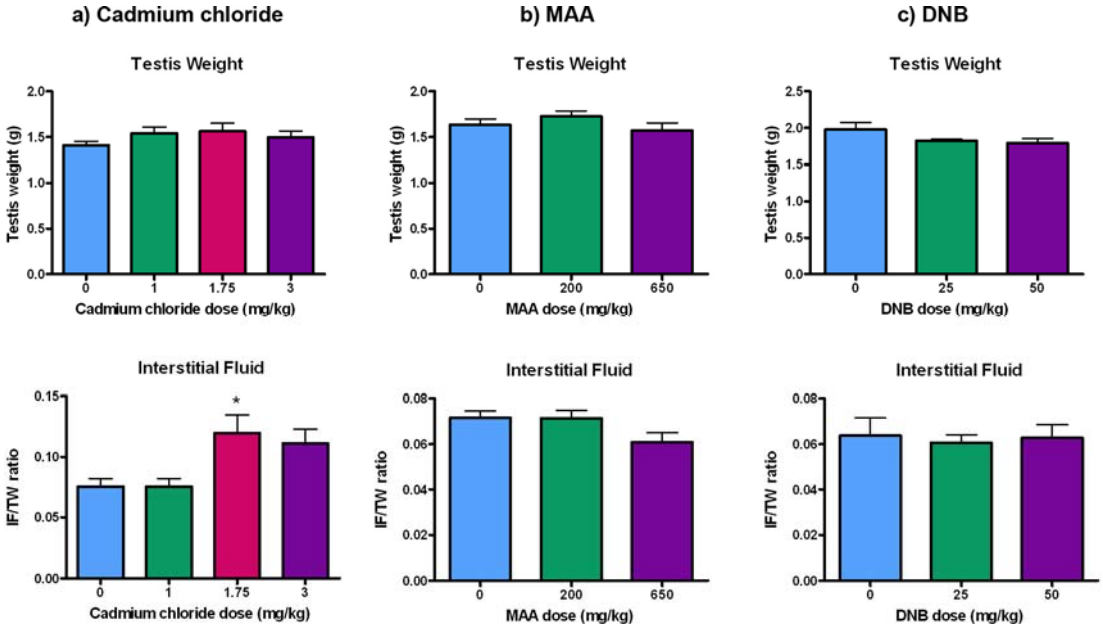


Figure 5-2 Testis Weights and Interstitial Fluid Weights expressed as ratio of testis weight (TW) for each dose group from a) Cadmium chloride study, b) MAA study and c) DNB study (mean with SEM). n=8 samples/group except 0mg/kg MAA where n=11. Statistical analysis, one-way ANOVA followed by Tukey post-test. * $p<0.05$ compared to control samples.

5.2.4 Immunohistochemistry & Immunofluorescence

Immunohistochemistry with the general antibody to seminiferous tubule proteins and an antibody to the germ cell protein DAZL, was carried out to investigate the damage caused by the toxicants to the testes. The DAB protocol described in section 3.5 was used. The presence of apoptotic cells was investigated by TUNEL immunohistochemistry (section 3.5.10).

Double immunofluorescence staining was used to investigate the integrity of the blood-testis barrier by staining for occludin/ZO-1, claudin-11/ZO-1, and N-cadherin/β-catenin, following the methods described in section 3.6. The biotin tracer (sections 3.3.6) was used for analysis of blood-testis barrier function, and detected by immunofluorescence as detailed in section 3.6.7.

5.2.5 Protein Analysis

Proteins present in interstitial fluid samples were separated by 1D gel electrophoresis (section 3.8), and analysed by Coomassie-based dye staining (section 3.9) to look at total protein, or by Western blotting with the general seminiferous tubule antibody (STCM), using the method described in section 3.10.

5.2.6 TaqMan® Q-PCR

Snap-frozen tissue collected from the cadmium chloride, MAA, and DNB studies was used for RNA isolation. cDNA was made from the isolated RNA samples, and used in the TaqMan® reaction with primers and probes for the blood-testis barrier genes, occludin, claudin-11, JAM-A, ZO-1, N-cadherin, and β-catenin. GAPDH was analysed as a control gene, and results are expressed relative to GAPDH expression. Detailed methods are described in section 3.11.

5.3 Results

5.3.1 Cadmium Chloride

5.3.1.1 Analysis of Damage caused by Cadmium Chloride Treatment

Cadmium chloride is a model testicular toxicant and is reported to disrupt the blood-testis barrier selectively at low doses. Four doses were selected, based on previous reports in the literature, to show a range of treatment effects, from mild to moderate.

Figure 5-3 clearly shows the dose response effect seen with the different doses. Immunohistochemistry with the antibody to seminiferous tubule conditioned medium detected a range of ST proteins and can be used to show the treatment effects at each dose. Testis sections from animals treated with 0.2mg/kg cadmium chloride or 1.0mg/kg cadmium chloride were comparable to control tissue, and suggest that there had been no effect of the treatment on the gross organisation of the STs. For this reason and in consideration of the 3Rs, the 0.2mg/kg dose was not included in repeat treatment studies. Testis sections from animals treated with either 1.75mg/kg or

3mg/kg cadmium chloride, and immunostained with the STCM antibody, showed disruption of the gross organisation of the STs, although the tubules themselves appeared intact. The effects seen with 3mg/kg cadmium chloride appeared more marked than those with the 1.75mg/kg dose.

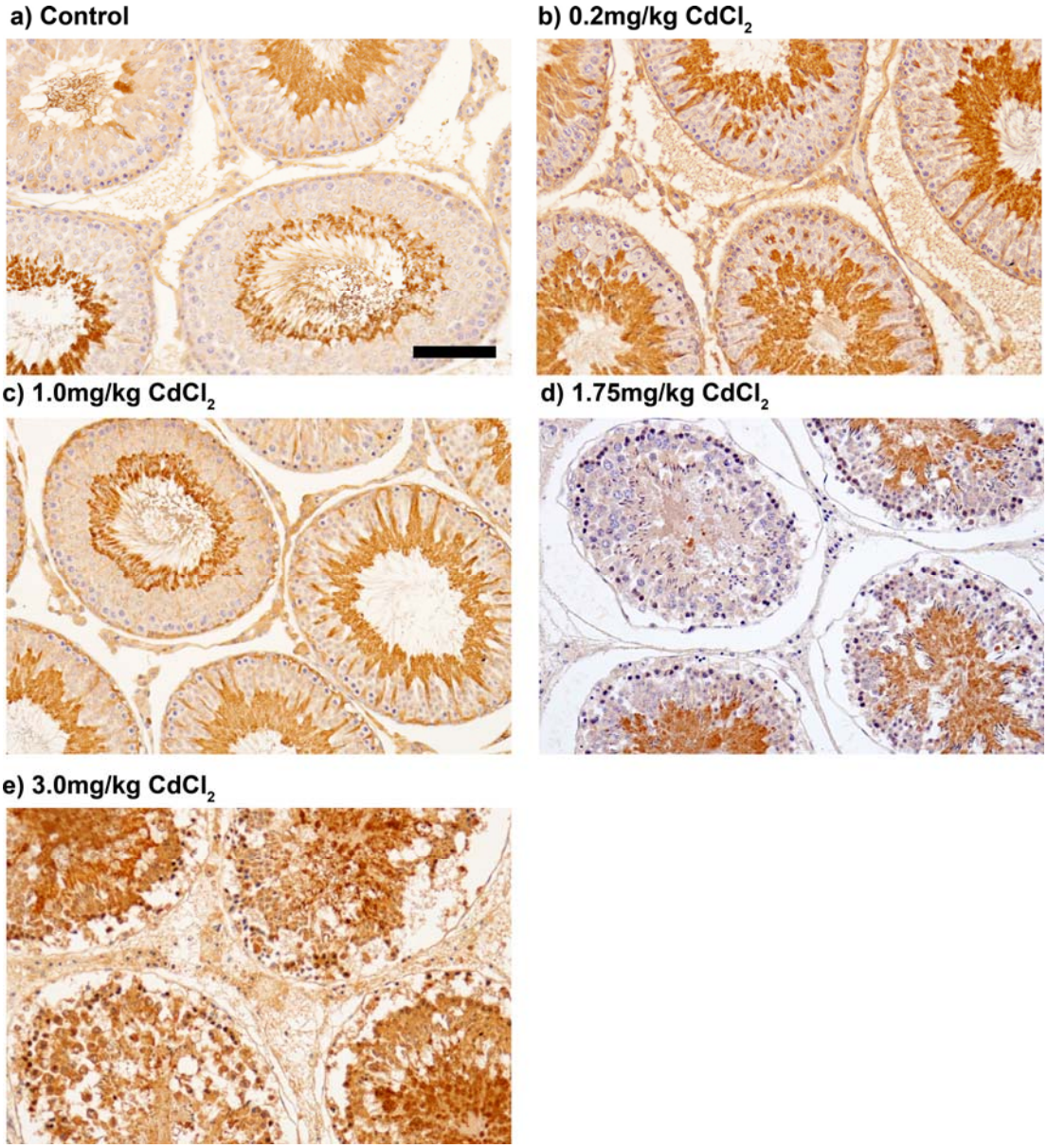


Figure 5-3 Testis sections from a) control (0.9% saline) rats and rats treated 24 hours earlier with b) 0.2mg/kg $CdCl_2$, c) 1.0mg/kg $CdCl_2$, d) 1.75mg/kg $CdCl_2$ or e) 3.0mg/kg $CdCl_2$, immunostained with an antibody to seminiferous tubule conditioned medium (brown) and counterstained with haematoxylin (blue). Scale bar represents 100µm.

5.3.1.2 Analysis of Protein Leakage

The STCM antibody was also used to detect general changes in the proteins present in interstitial fluid collected from rats treated with 1.0, 1.75 and 3.0mg/kg $CdCl_2$ using Western blotting. Figure 5-4a shows a representative Western blot, with two samples from each dose group run with the STCM antibody. A total of four blots were run to ensure all samples collected were used in the quantification analysis (n=7 for control, n=5 for 1mg/kg and n=8 for 1.75mg/kg and 3mg/kg). Quantification for two protein bands on the Western blots is shown in Figure 5-4b.

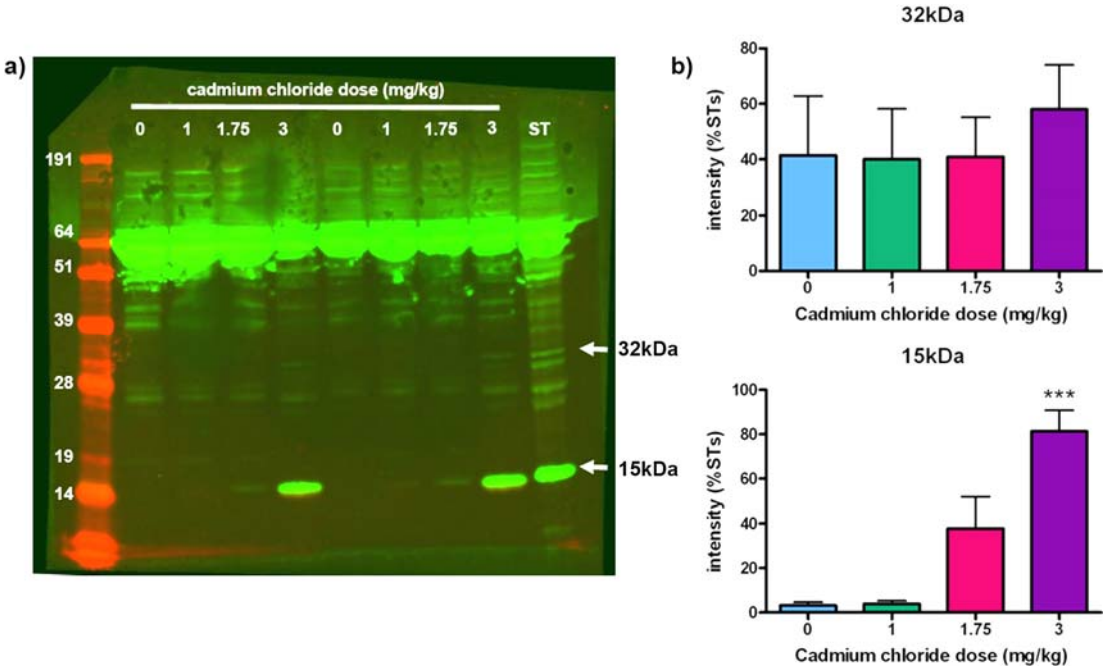


Figure 5-4 a) Representative Western Blot with antibody to STCM, showing interstitial fluid samples from control rats and animals treated with 1, 1.75 or 3.0mg/kg $CdCl_2$ and control homogenised seminiferous tubules (ST). Arrows indicate examples of protein bands quantified. b) Graphs showing quantification of 32kDa and 15kDa bands (mean with SEM), with values taken from samples run on four separate Western blots, expressed as a percentage of STs (n=7 for control, n=5 for 1mg/kg and n=8 for 1.75mg/kg and 3mg/kg). Statistical analysis, one-way ANOVA followed by Tukey post-test. *** $p<0.001$ compared to control samples.

As shown previously (section 4.3.1) the STCM antibody detected a large number of proteins. These could be germ cell derived, Sertoli cell derived or peritubular cell derived. In order to investigate the hypothesis that proteins leak out of STs into IF

under toxicological insult, control STs were run on every Western blot as a comparison and in order to identify the presence of ST derived proteins in IF samples.

Figure 5-4a shows a clear difference in the number of proteins present in IF from rats treated with 3mg/kg cadmium chloride, compared to IF from control rats. Two protein bands (32kDa, and 15kDa) were selected for quantification because they were present in the control ST sample, and because they appeared to show a dose response effect across IF samples. Despite observation of the band for the 32kDa protein only being present in the 3mg/kg $CdCl_2$ IF sample on the Western blot, the graph in Figure 5-4b, suggests that there is no significant difference in the presence of this protein across the $CdCl_2$ doses. This difference between observation and quantification, illustrates the importance of using a method for quantification and combining results from as many samples as possible run on different gels.

The second protein band with a size of 15kDa showed a different result when quantified. The Western blot shown in Figure 5-4a suggested a dose response effect of $CdCl_2$ in inducing leakage/presence of this protein in IF samples. The quantification graph in Figure 5-4b showed a significantly greater amount of this protein present in IF samples from animals treated with 3mg/kg $CdCl_2$ compared to control IF. This graph also suggested that there was more 15kDa protein present in IF from animals treated with 1.75mg/kg $CdCl_2$ compared to control, although this difference was not statistically significant. The large 15kDa band seen on the blot in the control ST sample, suggested that this protein is leaking from the STs into IF following treatment with high doses (3mg/kg and to an extent 1.75mg/kg) of cadmium chloride.

Although the Western blot with the STCM antibody shows some differences in the proteins present in IF collected from each dose group, there are likely to be many more proteins present in the samples that are not detected by antibodies in the STCM, some of which may also be present at different levels in the different dose

groups, and like the 15kDa protein, may also appear to leak from STs into IF with high dose treatment of cadmium chloride. In order to investigate this, 1D protein gels were run, and stained with Gelcode, a Coomassie dye based stain, in order to look at all the proteins present in the samples.

Figure 5-5a shows a representative Coomassie stained gel, with IF samples from the four cadmium chloride dose groups. A comparison of this gel and the STCM Western blot (Figure 5-4), showed many more proteins being detected with the Coomassie stain. Again there were clear differences between IF samples from the different cadmium chloride dose groups and more proteins were observed in IF samples from the 1.75 and 3mg/kg dose groups, compared to IF collected from control animals. This suggests that the higher doses of cadmium chloride are causing an increase in the number of proteins present in IF, presumably due to leakage of proteins from the seminiferous tubules. A range of protein bands were quantified, using ImageQuant software (refer to section 3.9.1) and results for nine such bands in all samples run on a total of four gels are shown in Figure 5-4b. Bands were selected for quantification due to ease of quantification, and their presence in the control ST sample as this ensured that they could have leaked from STs into IF.

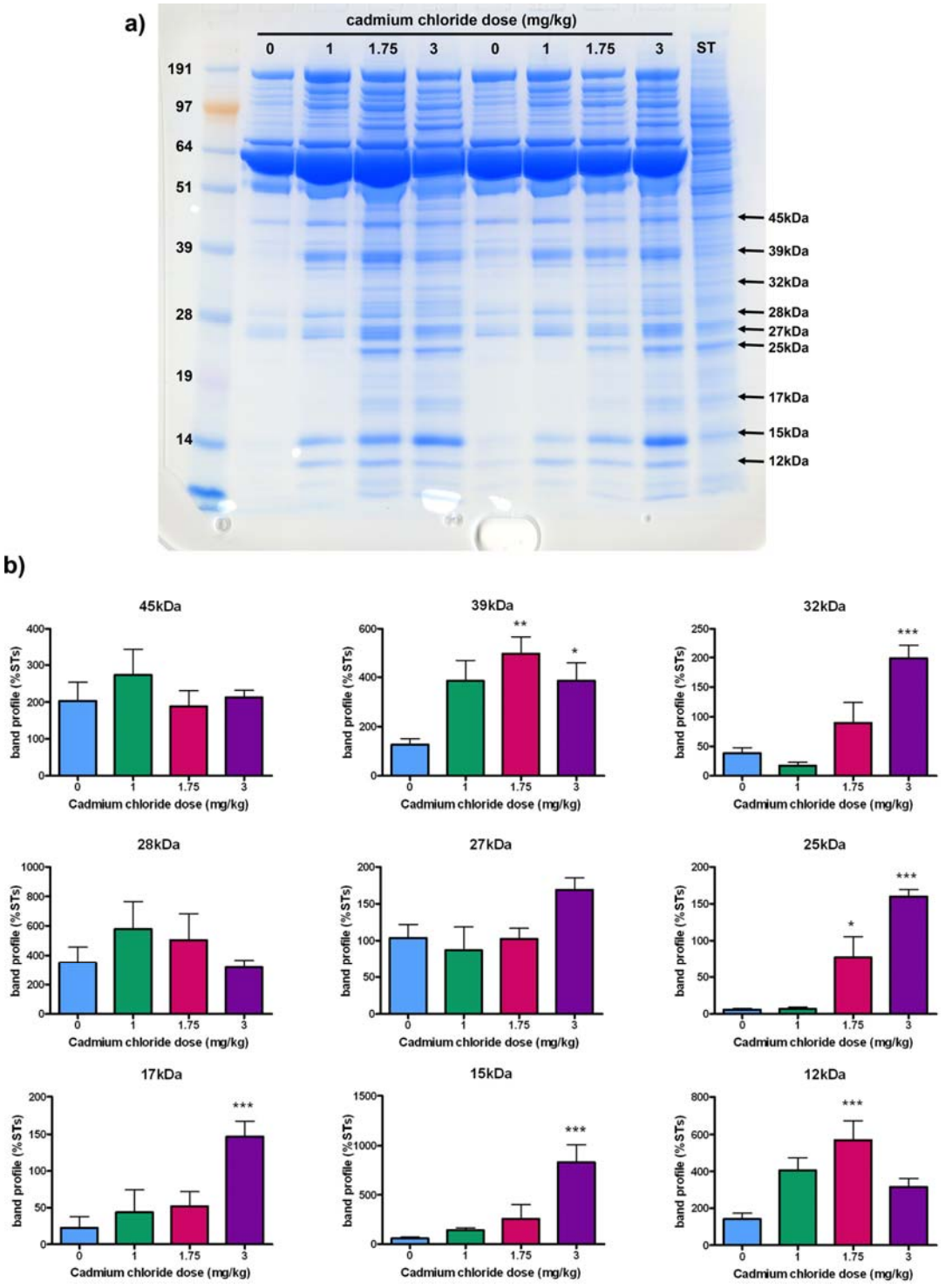


Figure 5-5 a) Representative Coomassie gel, showing interstitial fluid samples from control rats and animals treated with 1, 1.75 or 3.0mg/kg $CdCl_2$ and control homogenised seminiferous tubules (ST). Arrows indicate examples of protein bands quantified. b) Graphs showing quantification of indicated bands (mean with SEM), with values taken from samples run on four separate gels, expressed as a percentage of STs (n=8 samples/dose group, except 1mg/kg $CdCl_2$ where n=5). Statistical analysis, one-way ANOVA followed by Tukey post-test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, compared to control samples.

The graphs show that there were differences in IF protein levels across the $CdCl_2$ doses with some of the selected proteins (39kDa, 32kDa, 25kDa, 17kDa, 15kDa and 12kDa), whereas for others (45kDa, 28kDa, and 27kDa) there did not appear to be much change across the dose groups. Four proteins were present in significantly higher amounts in IF samples from animals treated with 3mg/kg $CdCl_2$, compared to control IF samples. These were 32kDa, 25kDa, 17kDa, and 15kDa in size, and these also showed an increase in IF protein levels with the 1.75mg/kg dose as well, although this was not statistically significant in all cases. The presence of these proteins in the control ST samples again suggests that these are ST proteins, which have leaked out into the IF following administration of high dose cadmium chloride.

The final two proteins, 39kDa, and 12kDa, showed a different pattern across the $CdCl_2$ dose groups, with a significantly higher (compared to control) amount present in IF from animals treated with 1.75mg/kg $CdCl_2$. With both proteins there was, however, no statistically significant difference in concentration across the three dose groups. This suggests that there could be an increase in concentration of these proteins following any dose of cadmium chloride or there could be a dose response up to 1.75mg/kg, but perhaps the same amount (or more) is leaking out following treatment with 3mg/kg $CdCl_2$ and then leaking out of the IF, possibly into the blood. This is probably unlikely as it does not appear to be happening with the other proteins quantified, and the two proteins in question are different sizes.

5.3.1.3 Analysis of Blood-Testis Barrier Integrity

To investigate whether the $CdCl_2$-induced protein leakage was due to loss of integrity of the blood-testis barrier (BTB), the tight junctions, adherens junctions and overall function of the BTB were assessed using immunofluorescent techniques.

Figure 5-6 shows co-localisation of the tight junction protein, occludin, and adaptor protein, ZO-1, at the site of the BTB in control tissue (panel a). Following treatment with 0.2mg/kg or 1.0mg/kg cadmium chloride, the tight junctions still appeared to be

intact, as shown by co-localisation of occludin and ZO-1 at the site of the BTB (panels b, c). In panels d and e, showing testis sections from animals treated with 1.75mg/kg and 3.0mg/kg $CdCl_2$ respectively, neither occludin nor ZO-1 could be identified at the site of the BTB. This suggests that the integrity of the BTB has been disturbed, as least in part via loss of occludin and ZO-1 from the tight junctions.

A further tight junction protein, claudin-11 was also assessed to confirm whether other components of the BTB had been disturbed following high dose cadmium chloride treatment. Figure 5-7 shows claudin-11 and ZO-1 localised to the site of the BTB in control tissue and tissue from animals treated with 1.0mg/kg $CdCl_2$. The variations seen were discussed in chapter 4. With testis sections from animals treated with 1.75mg/kg and 3.0mg/kg $CdCl_2$, some green staining (ZO-1) was observed although it is hard to tell whether this represents background or true staining. Either way, ZO-1 did not appear to be localised to the site of the BTB, as was seen with the control tissue. Claudin-11 was also no longer localised to the site of the BTB following treatment with 1.75mg/kg or 3.0mg/kg $CdCl_2$.

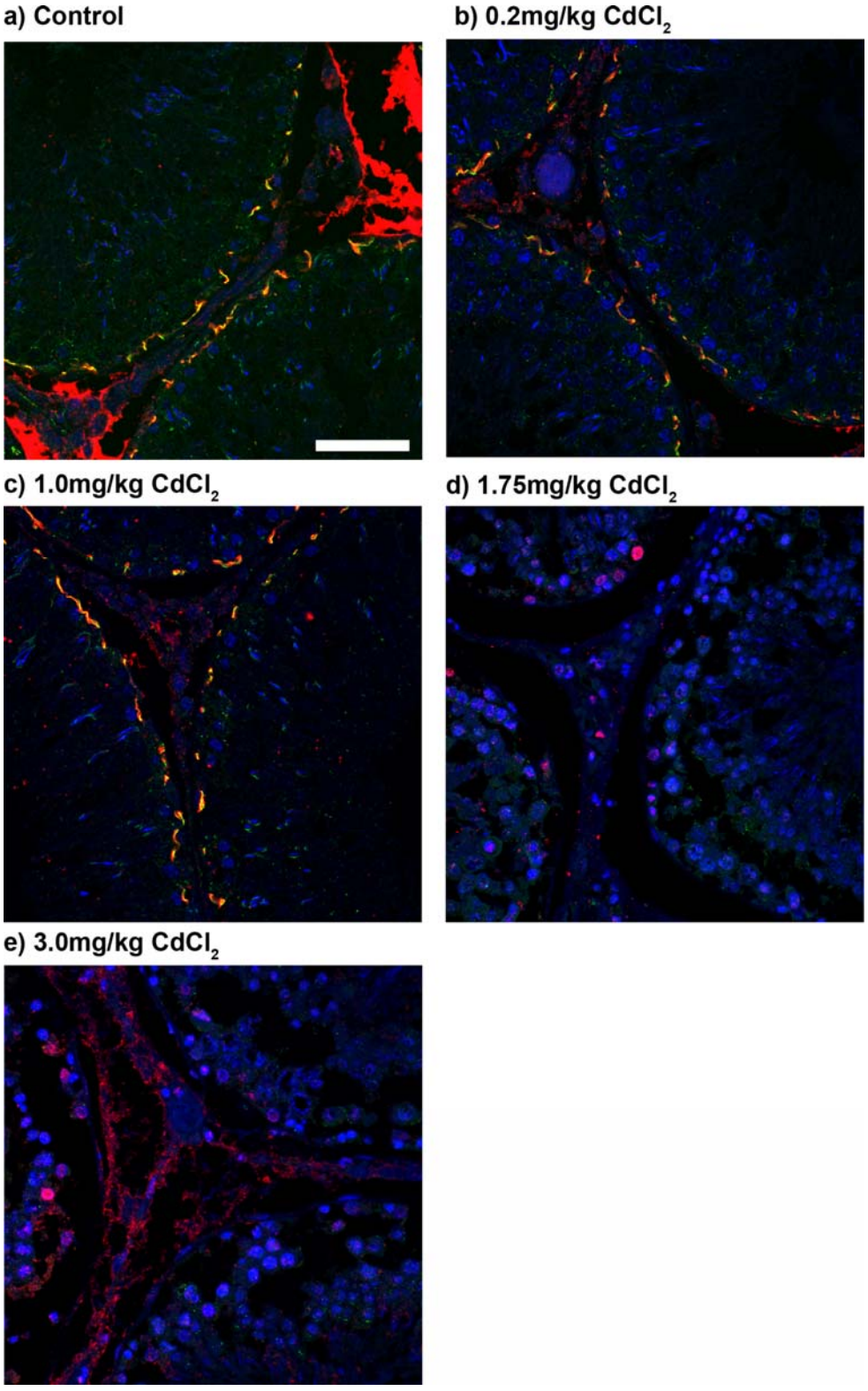


Figure 5-6 Co-localisation of ZO-1 (green) and occludin (red) with Dapi nuclear counterstain, in testis sections from a) control (0.9% saline) rats and rats treated 24 hours earlier with b) 0.2mg/kg $CdCl_2$, c) 1.0mg/kg $CdCl_2$, d) 1.75mg/kg $CdCl_2$ or e) 3.0mg/kg $CdCl_2$. Scale bar represents 50µm.

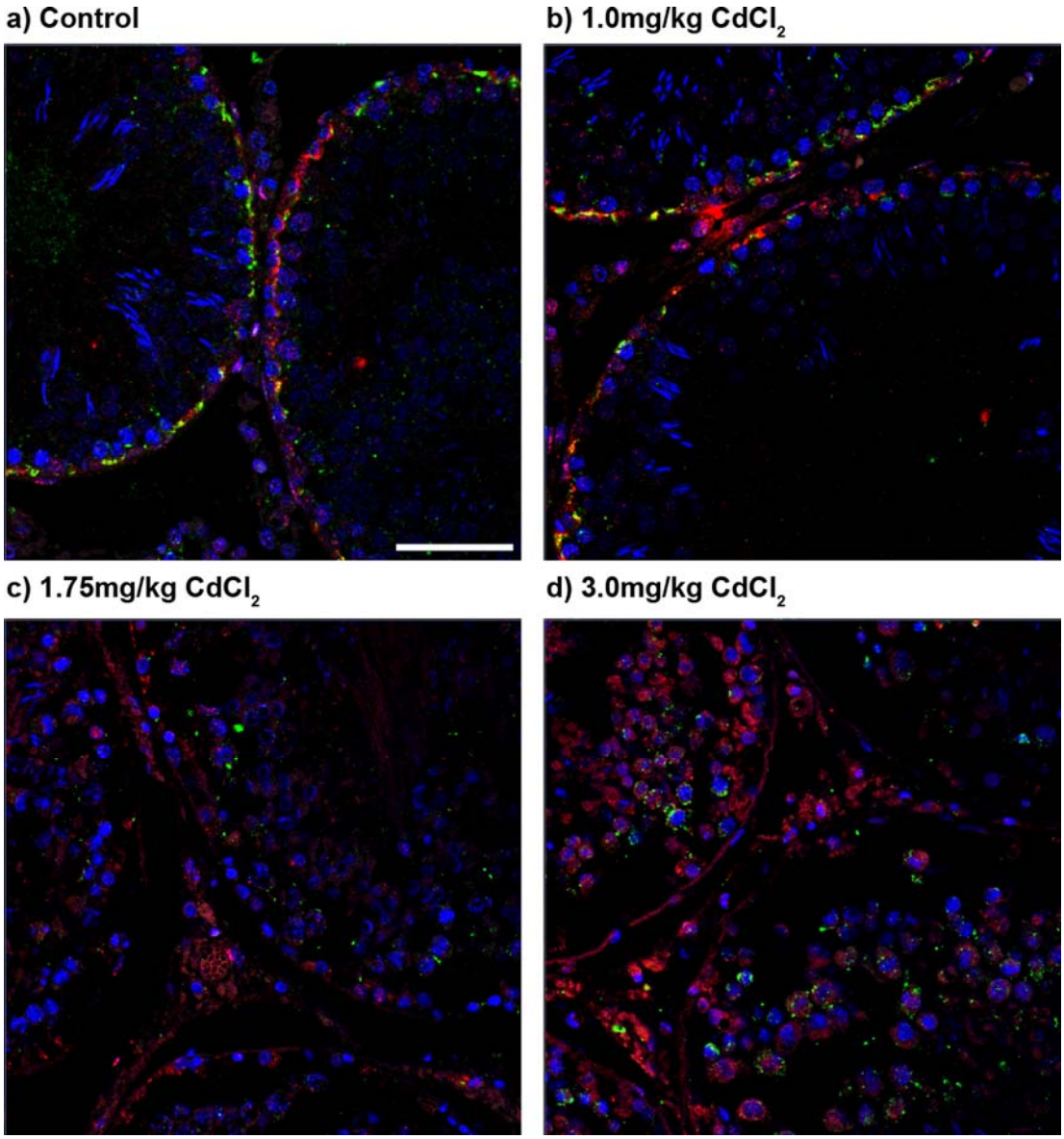


Figure 5-7 Co-localisation of ZO-1 (green) and claudin-11 (red) with Dapi nuclear counterstain in testis sections from a) control (0.9% saline) rats and rats treated 24 hours earlier with b) 1.0mg/kg $CdCl_2$, c) 1.75mg/kg $CdCl_2$ or d) 3.0mg/kg $CdCl_2$. Scale bar represents 50µm.

These results support the results found with occludin and ZO-1 (Figure 5-6), and together they suggest that the integrity of the tight junctions in the BTB has been disturbed following treatment with 1.75mg/kg or 3.0mg/kg $CdCl_2$. Although, there are many other tight junction proteins present in the BTB, an assessment of the three

proteins investigated seems sufficient to allow reasonable conclusions to be drawn. Two adherens junction proteins, N-cadherin and β-catenin, were also investigated to assess whether further components, other than the tight junctions, are disrupted in the BTB.

N-cadherin is an adherens junction protein, and can be clearly seen in red at the site of the BTB in control tissue in Figure 5-8a. β-catenin, an adherens junction adaptor protein can also be seen in Figure 5-8a localised around the nuclei of spermatocytes in the vicinity of the BTB (variation in staining patterns described in chapter 4). A similar staining pattern was seen in testis sections from animals treated with 1.0mg/kg $CdCl_2$. However, with testis sections from animals treated with 1.75mg/kg or 3.0mg/kg $CdCl_2$, this staining pattern was disrupted (Figure 5-8c,d). Thus, although some immunostaining for N-cadherin and β-catenin was evident localised around the area of the BTB, no distinct staining pattern along the site of the BTB was evident as was evident in control tissue. These results agree with the tight junction proteins previously investigated, and suggest that the adherens junctions at the site of the BTB are disrupted following treatment with 1.75mg/kg or 3.0mg/kg $CdCl_2$.

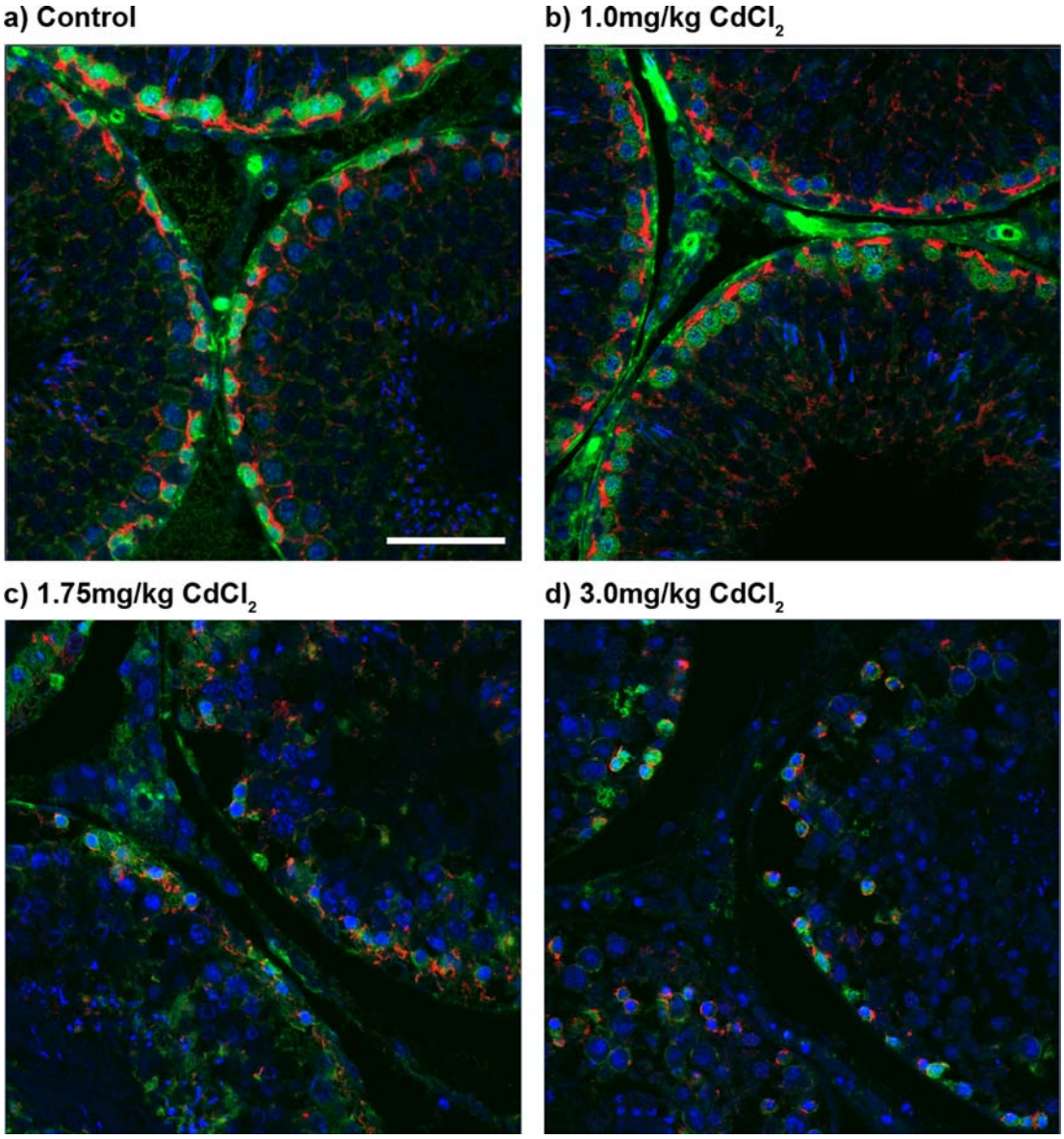


Figure 5-8 Co-localisation of β-catenin (green) and N-cadherin (red) with Dapi nuclear counterstain in testis sections from a) control (0.9% saline) rats and rats treated 24 hours earlier with b) 1.0mg/kg $CdCl_2$, c) 1.75mg/kg $CdCl_2$ or d) 3.0mg/kg $CdCl_2$. Scale bar represents 50μm.

A final immunofluorescent method to assess whether the BTB is functional was investigated, using injections of a biotin tracer and a fluorescent detection system. Figure 5-9, panel a, shows the biotin tracer localised to the interstitum, and surrounding spermatocytes on the interstitial side of the BTB (shown by the presence of occludin). The BTB was fully functional in control tissue, because as the tracer is

not membrane permeable, it cannot penetrate into the tubule if the BTB is functional. In animals treated with 1.0mg/kg $CdCl_2$, a similar staining pattern was seen, agreeing with the results seen with the specific junction proteins and showing that the BTB is functional in the testis. A similar pattern was again seen with tissue from animals treated with 1.75mg/kg $CdCl_2$ (Figure 5-9c), which contrasts with results for specific proteins in the BTB discussed above. This disparity could be due to the section of the testis used for the immunostaining – damage and disruption of the testis could have occurred in certain areas away from where the tissue was sectioned. Also this is an intermediate dose and some tubules could have been affected whereas others could appear normal.

With the 3.0mg/kg $CdCl_2$ treatment, no occludin was detected, as shown above, and the biotin tracer had penetrated the seminiferous tubule (Figure 5-9d). This agrees with the other results above with this dose and the individual junction proteins investigated, and further supports the idea that the BTB has been disrupted both structurally and functionally following treatment with 3.0mg/kg $CdCl_2$.

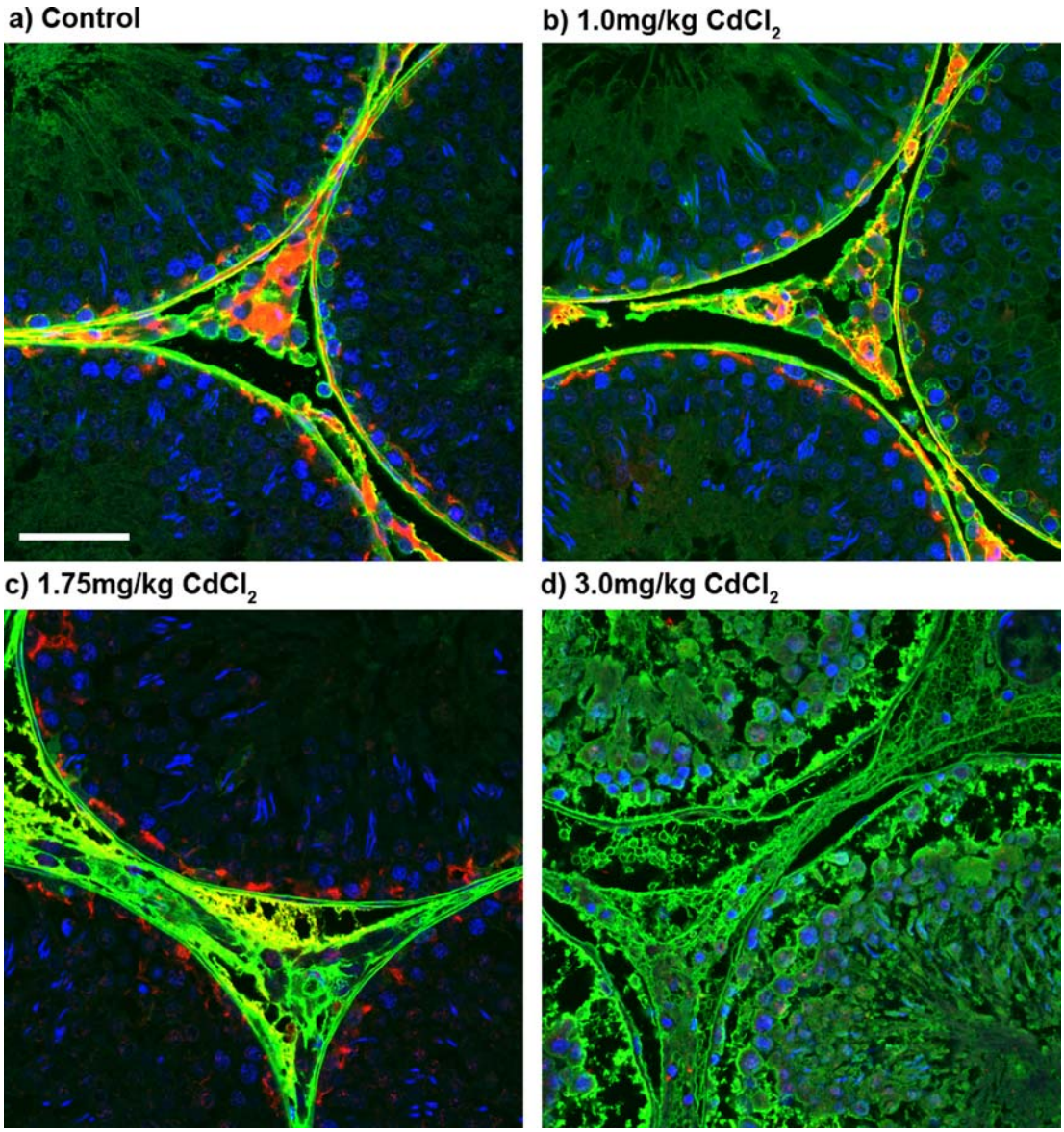


Figure 5-9 Detection of biotin tracer (green) and occludin (red) with Dapi nuclear counterstain in testis sections from a) control (0.9% saline) rats and rats treated 24 hours earlier with b) 1.0mg/kg $CdCl_2$, c) 1.75mg/kg $CdCl_2$ or d) 3.0mg/kg $CdCl_2$. Scale bar represents 50µm.

Analysis of mRNA expression of the BTB genes, occludin, claudin-11, JAM-A, ZO-1, N-cadherin, and β-catenin is shown in Figure 5-10. There was a significant decrease in the expression of the gene for the tight junction protein, occludin, relative to GAPDH expression, following treatment with 3mg/kg $CdCl_2$, this agrees with results for occludin protein visualised with immunofluorescence. A significant reduction in expression of claudin-11, JAM-A and ZO-1, relative to GAPDH

expression was also observed following treatment with 1.75mg/kg $CdCl_2$, supporting the immunofluorescent data as claudin-11 and ZO-1 proteins were all depleted following treatment with 1.75mg/kg $CdCl_2$ (see above). These results are, however, surprising as the protein data suggests that there should also be a decrease in expression of these tight junction genes following treatment with 3mg/kg $CdCl_2$, and this was not observed (Figure 5-10).

No differences in mRNA expression were seen with either of the adherens junction genes investigated, although there were downward trends in the $CdCl_2$ treated animals. Some variability in the data was noted, and results from one animal in the $CdCl_2$ high dose group were excluded as the histological analysis of one of the testes showed that the treatment had not had an effect on the structure of the testis. No significant differences were noted in the expression of the other control genes, SPAG5 and 3βHSD (not shown). These results suggest that there is an effect on mRNA expression for all four tight junction proteins following treatment with either 1.75 or 3mg/kg cadmium chloride, although a larger sample size should probably be used to achieve more accurate results.

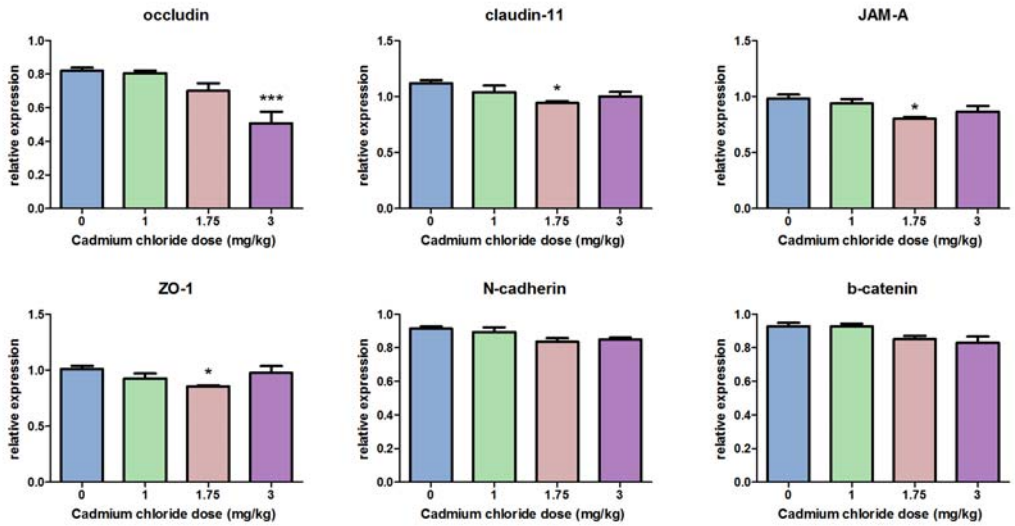


Figure 5-10 TaqMan® analysis of blood-testis barrier genes in testis samples collected from animals treated with cadmium chloride (mean with SEM). Expression relative to GAPDH expression. n=4 samples/dose group except 3mg/kg $CdCl_2$ where n=3. Statistical analysis, one-way ANOVA followed by Tukey post-test. * $p<0.05$, *** $p<0.001$ compared to control samples.

5.3.2 Methoxyacetic Acid

5.3.2.1 Analysis of Damage caused by MAA Treatment

Methoxyacetic acid (MAA) is another model testicular toxicant. It is well studied and known to induce death of pachytene spermatocytes by apoptosis leading to infertility (Tirado et al., 2003). MAA was selected as a second toxicant treatment to further investigate the protein leakage hypothesis and to investigate whether specific damage to germ cells also leads to leakage of proteins from STs into IF.

Two MAA doses were chosen based on previous reports in the literature, to show a range of treatment effects, from mild to moderate (Bartlett et al., 1988; Tirado et al., 2003). Figure 5-11 shows the damage caused by each dose of MAA treatment. Three immunohistochemistry markers were used. Panels a-c show the proteins detected by the general STCM antibody, panels d-f show the germ cell marker, DAZL, and panels g-i show the apoptosis marker TUNEL.

The general antibody to ST proteins showed that the 650mg/kg dose of MAA caused notable damage to the seminiferous tubules. This was most clearly visualised by examination of sections stained for the DAZL protein. DAZL is a germ cell protein that localises to pachytene spermatocytes in control tissue (Figure 5-11d). Following treatment with 650mg/kg MAA, the DAZL staining pattern was more sparse towards the basal compartment indicating a loss of pachytene spermatocytes, as expected with this dose of MAA. Treatment with 200mg/kg MAA showed an intermediate effect with loss of some pachytene spermatocytes but not to the extent seen with the 650mg/kg dose.

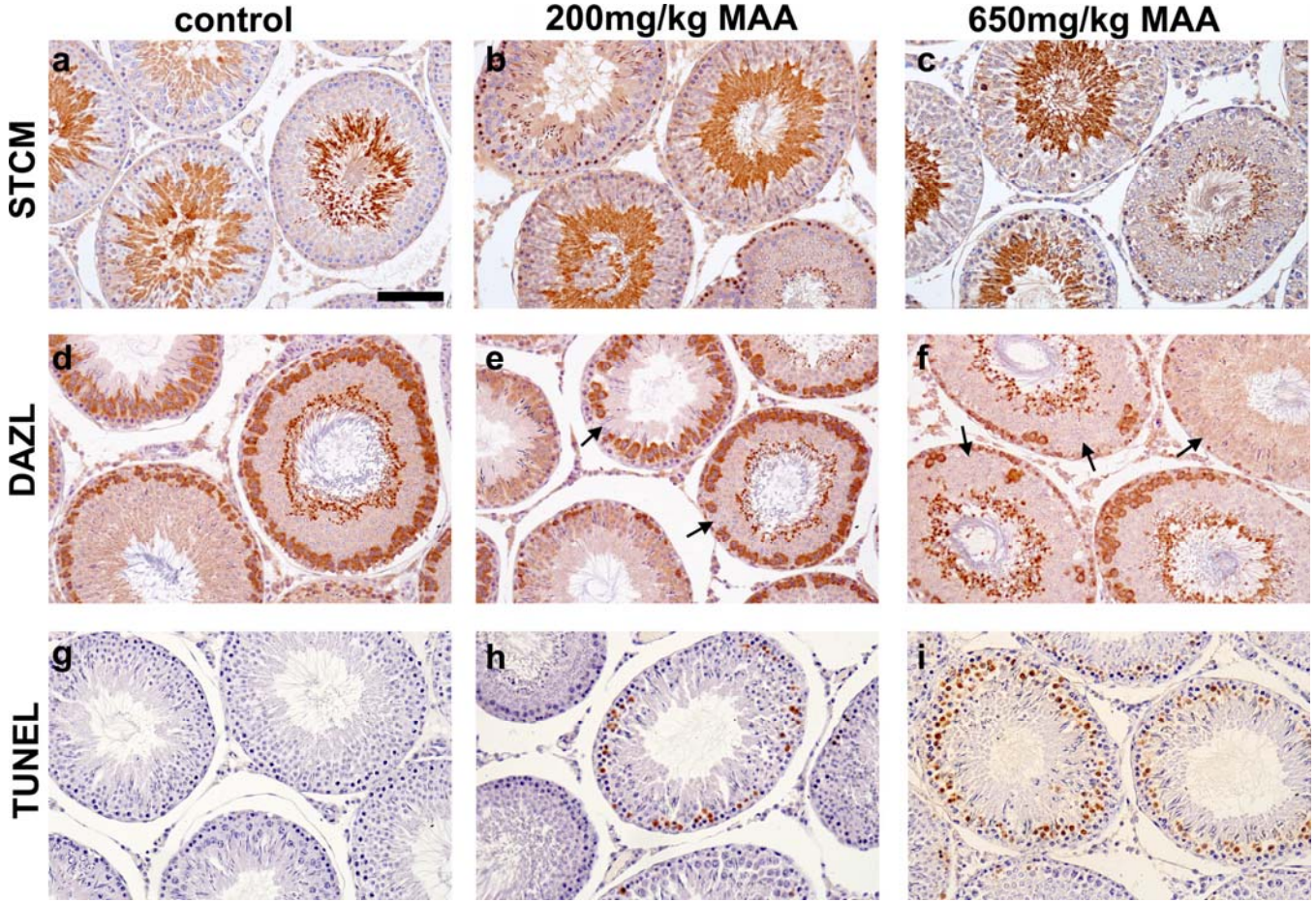


Figure 5-11 Testis sections from control (0.9% saline) rats and rats treated 24 hours earlier with 200mg/kg MAA or 650mg/kg MAA, immunostained with a-c) an antibody to seminiferous tubule conditioned medium (brown), d-f) DAZL (brown) and g-i) TUNEL (brown), all counterstained with haematoxylin (blue). Arrows indicate depletion of pachytene spermatocytes following MAA treatment. Scale bar represents 100µm.

The final marker used to illustrate the damage caused by MAA treatment in Figure 5-11 was TUNEL, a marker of apoptosis. Panels g-i show an increase in the number of apoptotic cells across the dose groups. Comparing this to the effect observed in panels d-f with an increasing loss of spermatocytes across the dose groups, it is likely that this loss of spermatocytes is caused by treatment induced apoptosis of the spermatocytes.

5.3.2.2 Analysis of Protein Leakage

The STCM antibody was also used to investigate protein leakage from STs into IF, following MAA treatment. Figure 5-12a shows a representative Western blot with two samples from each dose group run with the STCM antibody. A total of four blots were run to ensure all samples collected were used in the quantification analysis (n=10 for control, n=8 for treated). Quantification for two protein bands on the Western blots is shown in Figure 5-12b.

From the Western blots, there did not appear to be any major differences between the STCM antibody-detected proteins present in control IF when compared to IF collected from animals treated with 200mg/kg or 650mg/kg MAA. Control STs were run alongside IF samples to assess whether ST proteins were leaking into IF following toxicant treatment, and to allow normalisation of bands during quantification of bands on different Western blots.

Two proteins bands, at 27kDa and 15kDa, were quantified from this Western blot (Figure 5-12b). The graphs show no significant differences between the amount of these proteins present in control IF, compared to the amount present in IF from animals treated with 200mg/kg or 650mg/kg MAA and the values obtained probably represent background ‘noise’. However, there did appear to be a trend with the 15kDa protein for more present in IF from 650mg/kg MAA treated animals. As before, with the cadmium chloride studies, protein leakage was further investigated by running IF samples on Coomassie stained gels.

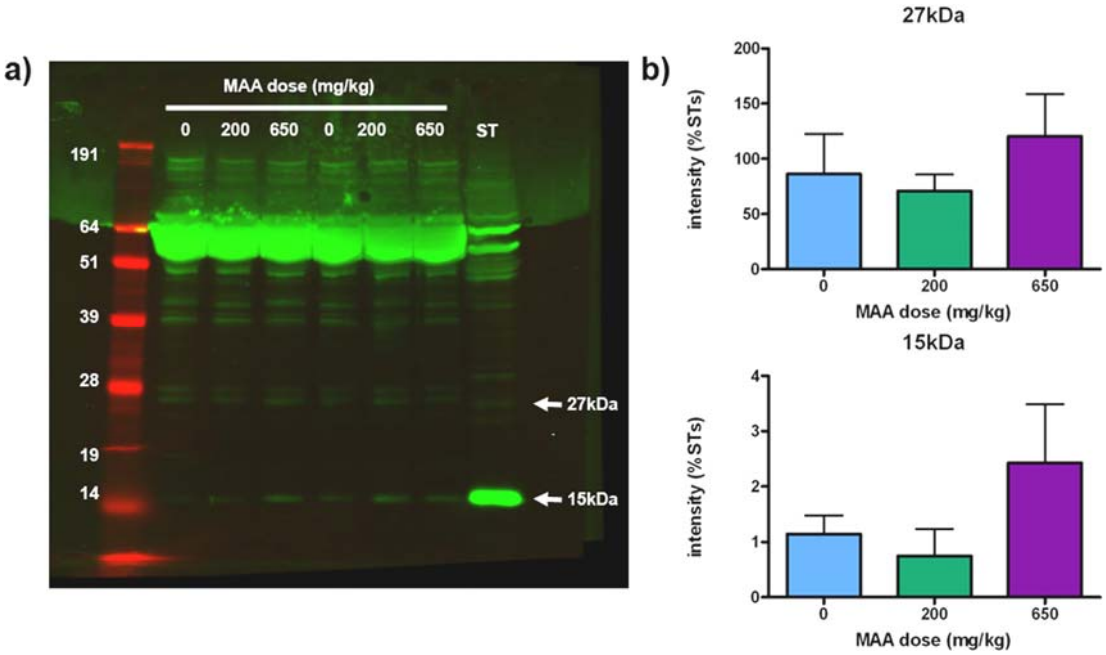


Figure 5-12 a) Representative Western blot with antibody to STCM, showing interstitial fluid samples from control rats and animals treated with 200 or 650mg/kg MAA and control homogenised seminiferous tubules (ST). Arrows indicate examples of protein bands quantified. b) Graphs showing quantification of 27kDa and 15kDa bands (mean with SEM), with values taken from samples run on four separate Western blots, expressed as a percentage of STs (n=10 for control, n=8 for treated). No significant differences following statistical analysis (one-way ANOVA followed by Tukey post-test).

Figure 5-13 shows a representative gel with IF samples from control animals, and those treated with either 200mg/kg or 650mg/kg MAA, and control STs for comparison between gels, and identification of ST proteins, stained with Gelcode Coomassie stain. As expected, many more proteins were identified on this gel compared to the Western blot in Figure 5-12. However, inspection of the bands on the gel also suggested that there were no major differences in proteins present in IF from control animals and those treated with 200mg/kg or 650mg/kg MAA. Six protein bands were quantified and the results are shown in Figure 5-13. Quantification indicated no significant differences between the amount of each protein present in IF from control animals or animals treated with 200 or 650mg/kg MAA.

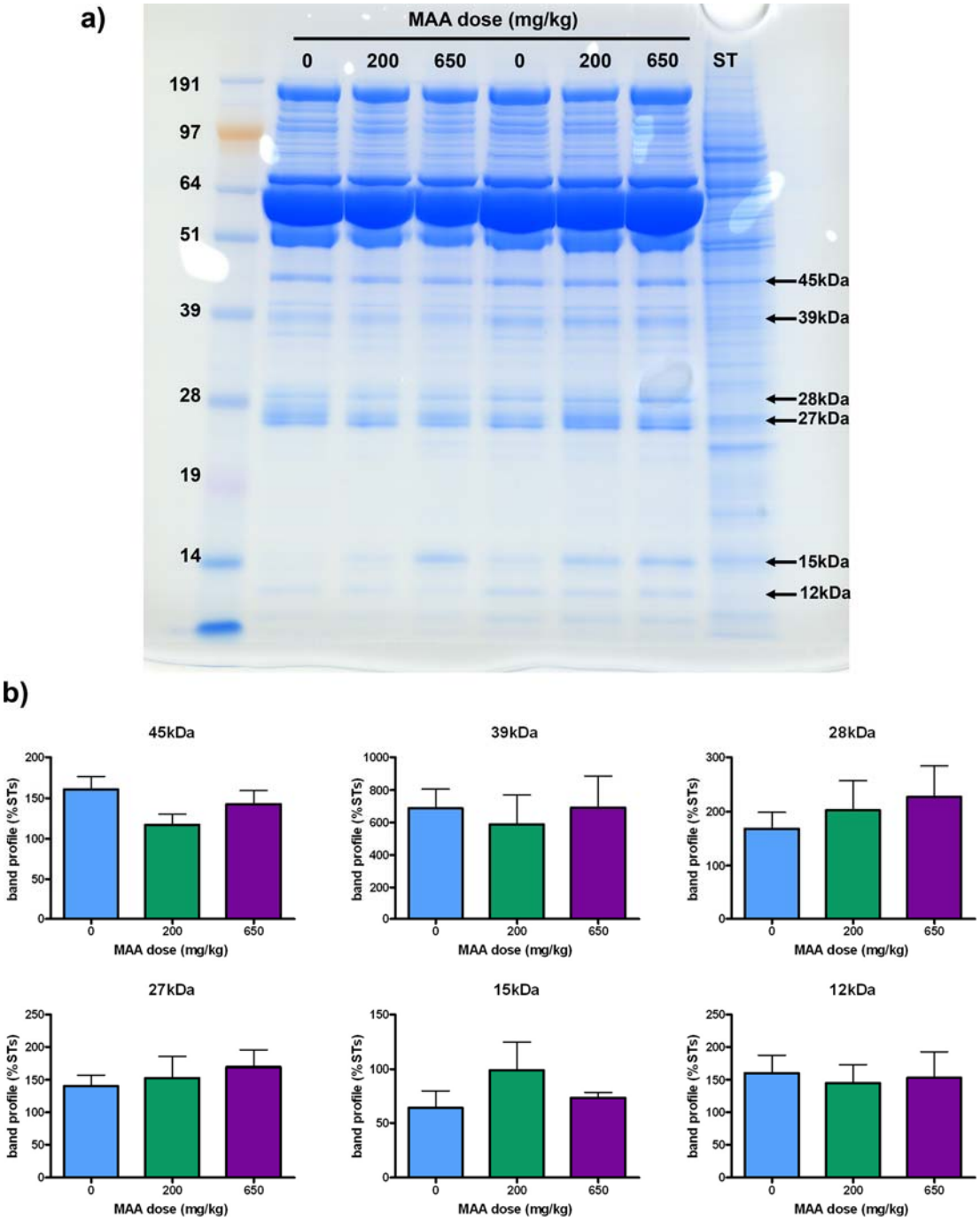


Figure 5-13 a) Representative Coomassie gel, showing interstitial fluid samples from control rats and animals treated with 200 or 650mg/kg MAA and control homogenised seminiferous tubules (ST). Arrows indicate examples of protein bands quantified. b) Graphs showing quantification of indicated bands (mean with SEM), with values taken from samples run on four separate gels, expressed as a percentage of STs (n=10 for control, n=8 for treated). No significant differences following statistical analysis (one-way ANOVA followed by Tukey post-test).

These interstitial fluid analyses suggest that proteins are not leaking out of STs into IF following degeneration and loss of pachytene spermatocytes following MAA treatment. Analysis of the integrity of the BTB was also carried out with MAA treated tissue to confirm whether the barrier was intact and therefore preventing proteins leaking out of the tubules.

5.3.2.3 Analysis of Blood-Testis Barrier Integrity

As with the cadmium chloride studies, integrity of the blood-testis barrier was investigated by immunofluorescent analysis of junction proteins and by using the biotin tracer. Figure 5-14 shows immunofluorescent co-localisation studies for the tight junction protein pairs, ZO-1 and occludin, and ZO-1 and claudin-11, and the adherens junction protein pair, β-catenin and N-cadherin. In panel a, ZO-1 and occludin can be seen to be co-localised at the site of the BTB in control tissue. In b and c, ZO-1 and occludin can, again, be seen co-localised at the site of the BTB, suggesting that the integrity of the tight junctions in the BTB has not been compromised by treatment with either 200mg/kg or 650mg/kg MAA. Results with ZO-1 and claudin-11 (shown in panels d-f), suggest the same conclusion, although the problems with this immunofluorescent staining combination as described previously (chapter 4) should be noted. The adherens junction proteins, β-catenin and N-cadherin, shown in panels g-i are located at the site of the BTB in the control tissue, as well as in tissue taken from animals treated with either 200mg/kg or 650mg/kg MAA. This agrees with the results seen with the tight junction proteins, and suggests that the BTB is still intact following treatment with either dose of MAA.

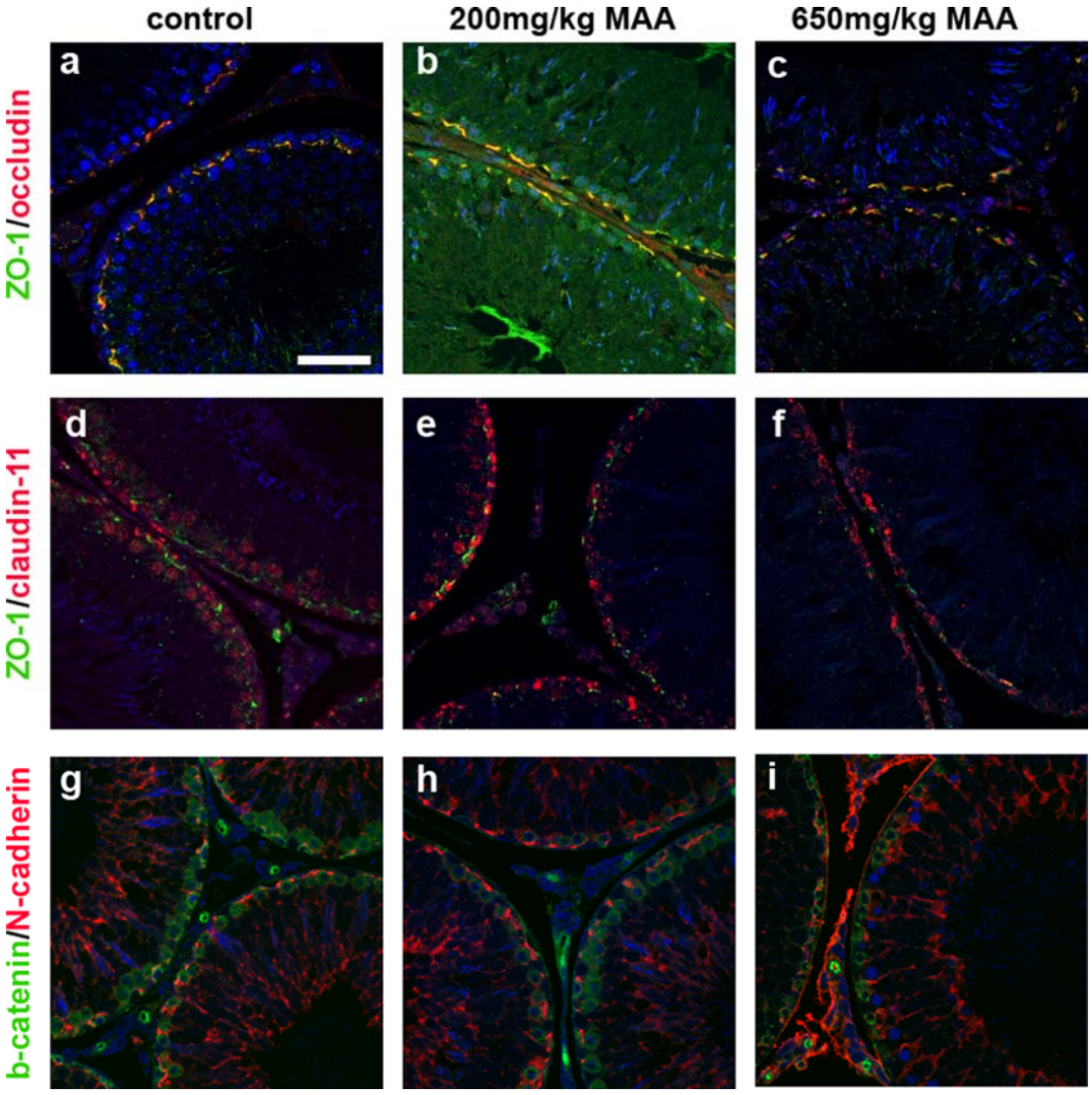


Figure 5-14 Testis sections from control (0.9% saline) rats and rats treated 24 hours earlier with 200mg/kg MAA or 650mg/kg MAA, showing co-immunofluorescence for a-c) ZO-1 (green) and occludin (red), d-f) ZO-1 (green) and claudin-11 (red) and g-i) β-catenin (green) and N-cadherin (red), all counterstained with dapi (blue). Scale bar represents 50μm.

The functionality of the BTB following MAA treatment was investigated using the biotin tracer. Figure 5-15 shows detection of the tracer, and the location of occludin and, therefore, the site of the BTB. In panel a, control tissue, the biotin tracer can be detected in the interstitum, and up to the BTB, showing that the barrier is functional. The same can be seen with testes collected from animals treated with either 200mg/kg or 650mg/kg MAA, showing that the BTB is fully functional in the tissue. This agrees with the results seen looking at the co-localisation of the individual

proteins, and suggests that treatment with MAA has not affected the integrity of the BTB.

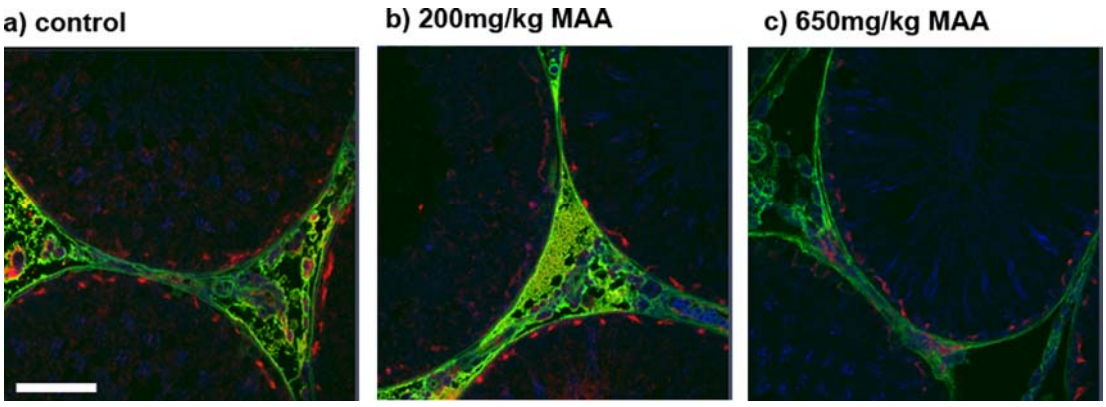


Figure 5-15 Detection of biotin tracer (green) and occludin (red) with Dapi nuclear counterstain, in testis sections from a) control (0.9% saline) rats and rats treated 24 hours earlier with b) 200mg/kg MAA, or c) 650mg/kg MAA. Scale bar represents 50µm.

Analysis of the genes for BTB proteins, occludin, claudin-11, JAM-A, ZO-1, N-cadherin and β-catenin, by TaqMan® PCR is shown in Figure 5-16. No significant differences in the number of mRNA copies for all six genes, relative to GAPDH expression was observed across the MAA dose groups. This supports the protein results shown above, and suggests that there are no differences in expression of the BTB proteins or their genes following treatment with either dose of MAA, despite the 650mg/kg dose clearly having a major adverse effect on pachytene spermatocytes, and the 200mg/kg MAA dose having a milder effect.

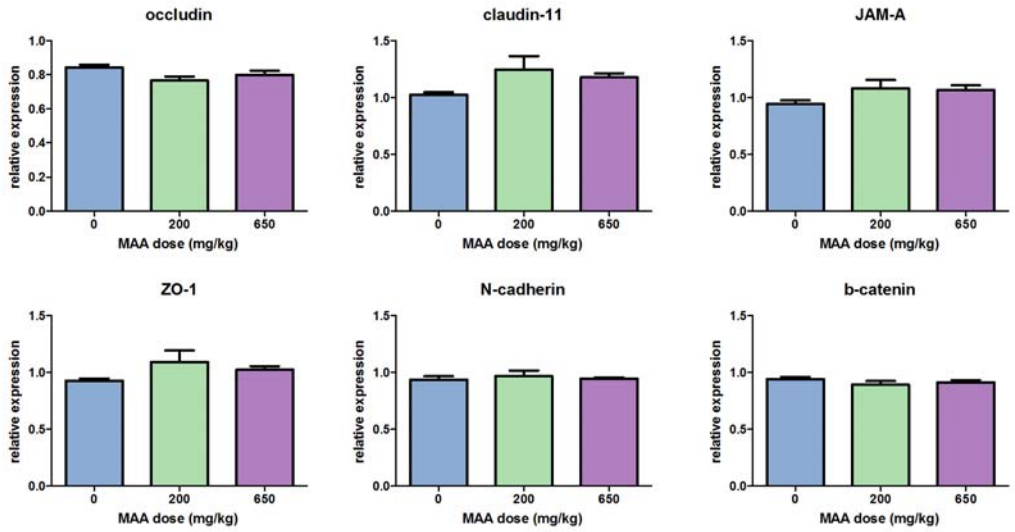


Figure 5-16 TaqMan® analysis of blood-testis barrier genes in testis samples collected from animals treated with MAA (mean with SEM). Expression relative to GAPDH expression. n=4 samples/dose group except 200mg/kg MAA for β-catenin where n=3. No significant differences following statistical analysis (one-way ANOVA followed by Tukey post-test).

5.3.3 1,3-dinitrobenzene

5.3.3.1 Analysis of Damage caused by DNB Treatment

1,3-dinitrobenzene (DNB) is another model testicular toxicant. It is known to specifically target Sertoli cells (Blackburn et al., 1988). DNB was selected as a third toxicant treatment to further investigate the protein leakage hypothesis and to investigate whether specific damage to Sertoli cells would lead to leakage of proteins from STs into IF.

Two DNB doses were chosen based on previous reports in the literature, to show a range of treatment effects, from mild to moderate. Figure 5-17 shows the damage caused by each treatment dose. As with MAA, three immunohistochemistry markers were used to evaluate treatment effects. Panels a-c show the proteins detected by the general STCM antibody, panels d-f show the germ cell marker, DAZL, and panels g-i show the apoptosis marker TUNEL.

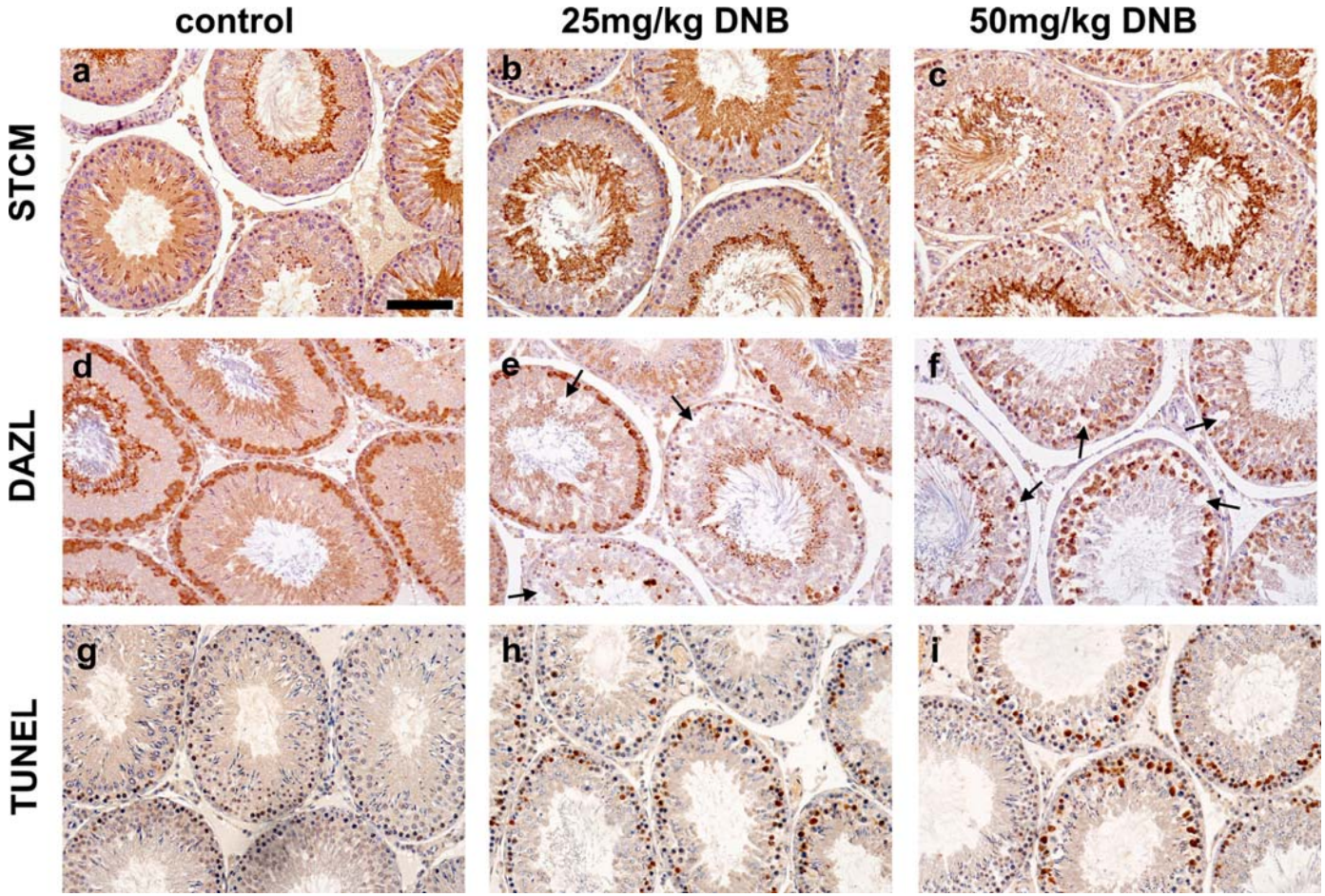


Figure 5-17 Testis sections from control (corn oil) rats and rats treated 24 hours earlier with 25mg/kg DNB or 50mg/kg DNB, immunostained with a-c) an antibody to seminiferous tubule conditioned medium (brown), d-f) DAZL (brown) or g-i) TUNEL (brown), all counterstained with haematoxylin (blue). Arrows indicate Sertoli cell vacuolation caused by DNB treatment. Scale bar represents 100μm.

The general antibody to ST proteins revealed evidence of damage to the organisation of the tubules following treatment with 50mg/kg DNB. However, the germ cell marker DAZL gave a better picture of the damage caused. A loss of spermatocytes was evident after treatment with either 25mg/kg DNB or 50mg/kg DNB (panels e,f). These images also showed damage via vacuolation of the Sertoli cells with both doses. Qualitative analysis of apoptotic cells (shown in panels g-i), identified by TUNEL staining, suggested an increase in the number of apoptotic cells after either DNB dose when compared to control.

5.3.3.2 Analysis of Protein Leakage

The leakage of proteins from STs into IF following DNB treatment was investigated using Western blots with the antibody to STCM, followed by further analysis with Coomassie stained gels. Figure 5-18 shows a representative Western blot with IF collected from animals treated with control, 25mg/kg or 50mg/kg DNB, run alongside control STs for protein band comparison, and probed with the general antibody to ST proteins. The blot shows that a similar number of proteins are detected in each IF sample, and at about the same level, except in the first 25mg/kg DNB sample where there appeared to be more of every protein detected. Because there appeared to be an increase in the amount of every protein, this suggests that perhaps slightly more total protein was loaded into this lane on the original gel. It is for this reason that all samples (n=8 samples/dose group) were run on Western blots, and selected protein bands were then quantified and expressed in the graphs in Figure 5-18b. The graphs for the 27kDa protein and 15kDa protein showed no significant differences in the amount of protein detected in IF from animals in either control or DNB treatment groups.

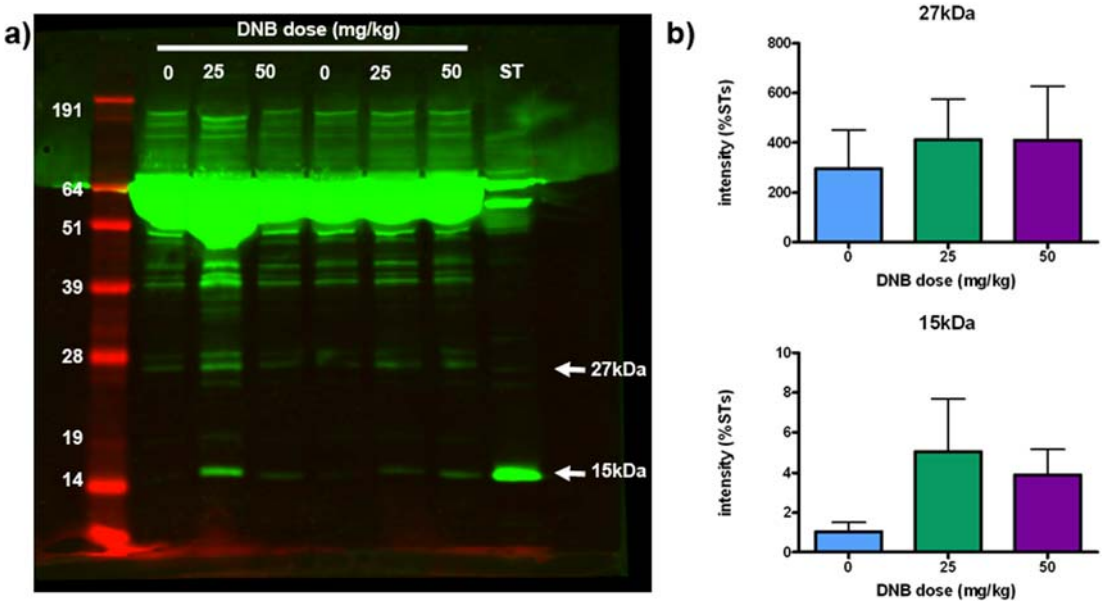


Figure 5-18 a) Representative Western blot with antibody to STCM, showing interstitial fluid samples from control rats and animals treated with 25 or 50mg/kg DNB and control homogenised seminiferous tubules (ST). Arrows indicate examples of protein bands quantified. b) Graphs showing quantification of 27kDa and 15kDa bands (mean with SEM), with values taken from samples run on four separate Western blots, expressed as a percentage of STs (n=8 samples/dose group). No significant differences following statistical analysis (one-way ANOVA followed by Tukey post-test).

Coomassie stained gels were also used to investigate whether proteins leaked from STs into IF following DNB treatment. Figure 5-19 shows a representative gel. As with the other treatments, many more proteins were identified with Coomassie staining, compared to Western blotting with the STCM antibody, although these proteins were also unknown. Six protein bands were quantified from the gel (and a further three gels, containing all the other IF samples collected from each animal treated in the DNB studies), and the results are shown in Figure 5-19b. The combined graphs showed no significant differences in the amount of any of the six proteins across the DNB dose groups. Particular attention should be paid to the proteins of sizes 15kDa and 12kDa which showed a dose related increase in IF in cadmium chloride treated animals (Figure 5-5).

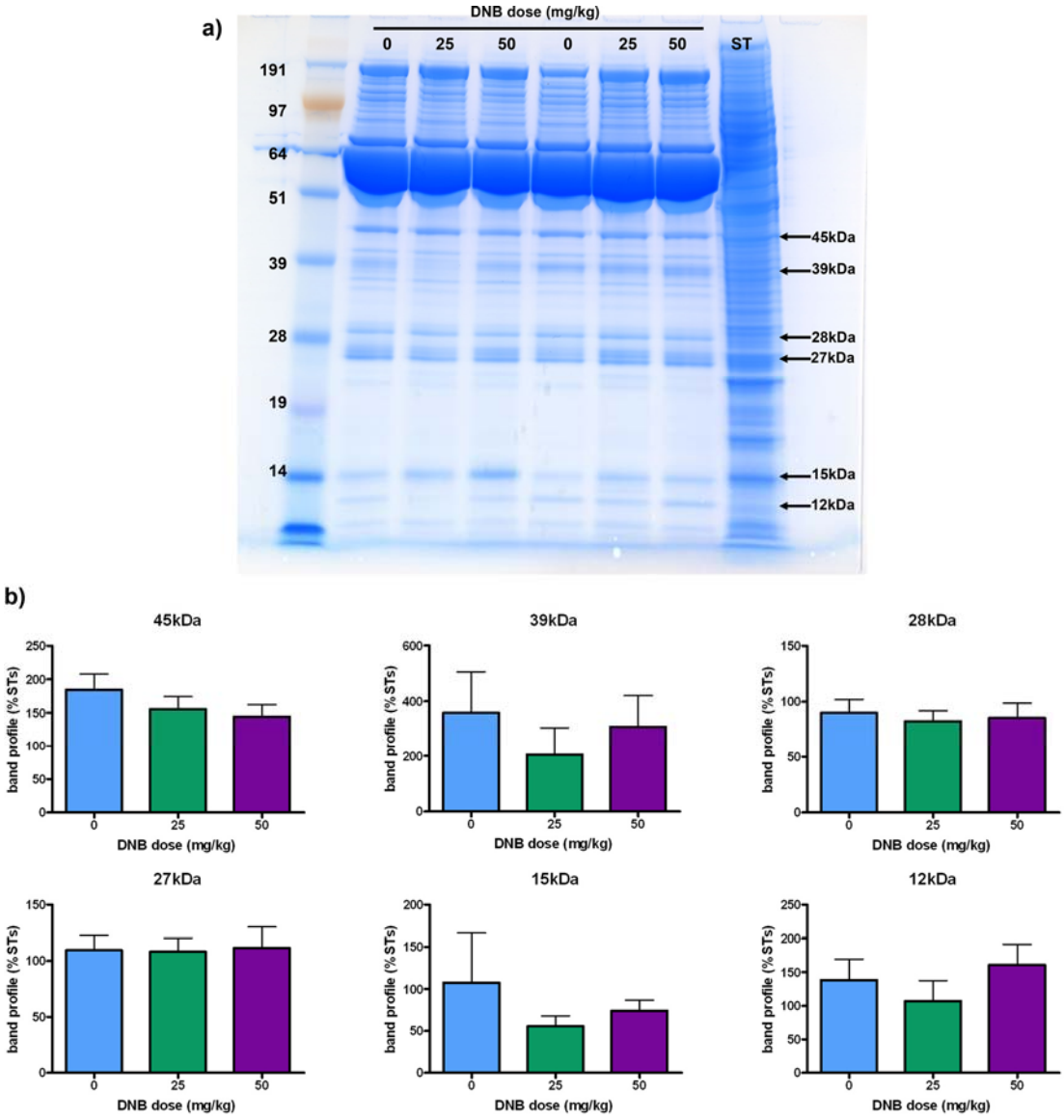


Figure 5-19 a) Representative Coomassie gel, showing interstitial fluid samples from control rats and animals treated with 25 or 50mg/kg DNB and control homogenised seminiferous tubules (ST). Arrows indicate examples of protein bands quantified. b) Graphs showing quantification of indicated bands (mean with SEM), with values taken from samples run on four separate gels, expressed as a percentage of STs (n=8 samples/dose group). No significant differences following statistical analysis (one-way ANOVA followed by Tukey post-test).

The results from the Coomassie gels and the Western blots with STCM antibody suggest that proteins are not leaking out of the seminiferous tubules into the interstitial fluid following damage to the Sertoli cells, and loss of spermatocytes caused by DNB treatment. Analysis of the integrity of the BTB was carried out to

confirm whether the barrier was still intact and functional following DNB treatment, and therefore preventing protein leakage out into the IF.

5.3.3.3 Analysis of Blood-Testis Barrier Integrity

The integrity of the BTB following DNB treatment was assessed by immunofluorescence studies of junction proteins, and by using the biotin tracer. Figure 5-20 panels a-c show the tight junction proteins ZO-1 and occludin. Both proteins can be seen to co-localise at the site of the BTB in control tissue, and in testes from animals treated with either 25mg/kg or 50mg/kg DNB, suggesting that the tight junctions have not been disrupted. A third tight junction protein, claudin-11 was also investigated. The results, shown in panels d-f, also showing the tight junction accessory protein ZO-1, suggest that claudin-11 is present in tubules across the dose groups, although ZO-1 and claudin-11 do not appear to co-localise at the BTB in every tubule. The problems with immunofluorescent detection of this tight junction protein pair were discussed in chapter 4, and due to these problems, the results seen with claudin-11 and in testes from DNB treated animals also suggested that the tight junctions are still intact. The adherens junction protein pair, β-catenin and N-cadherin, were also investigated following DNB treatment. Figure 5-20g-i shows the localisation of both proteins at the site of the BTB, and suggests no differences in the state of the adherens junctions between control tissue, and that collected from animals treated with either dose of DNB.

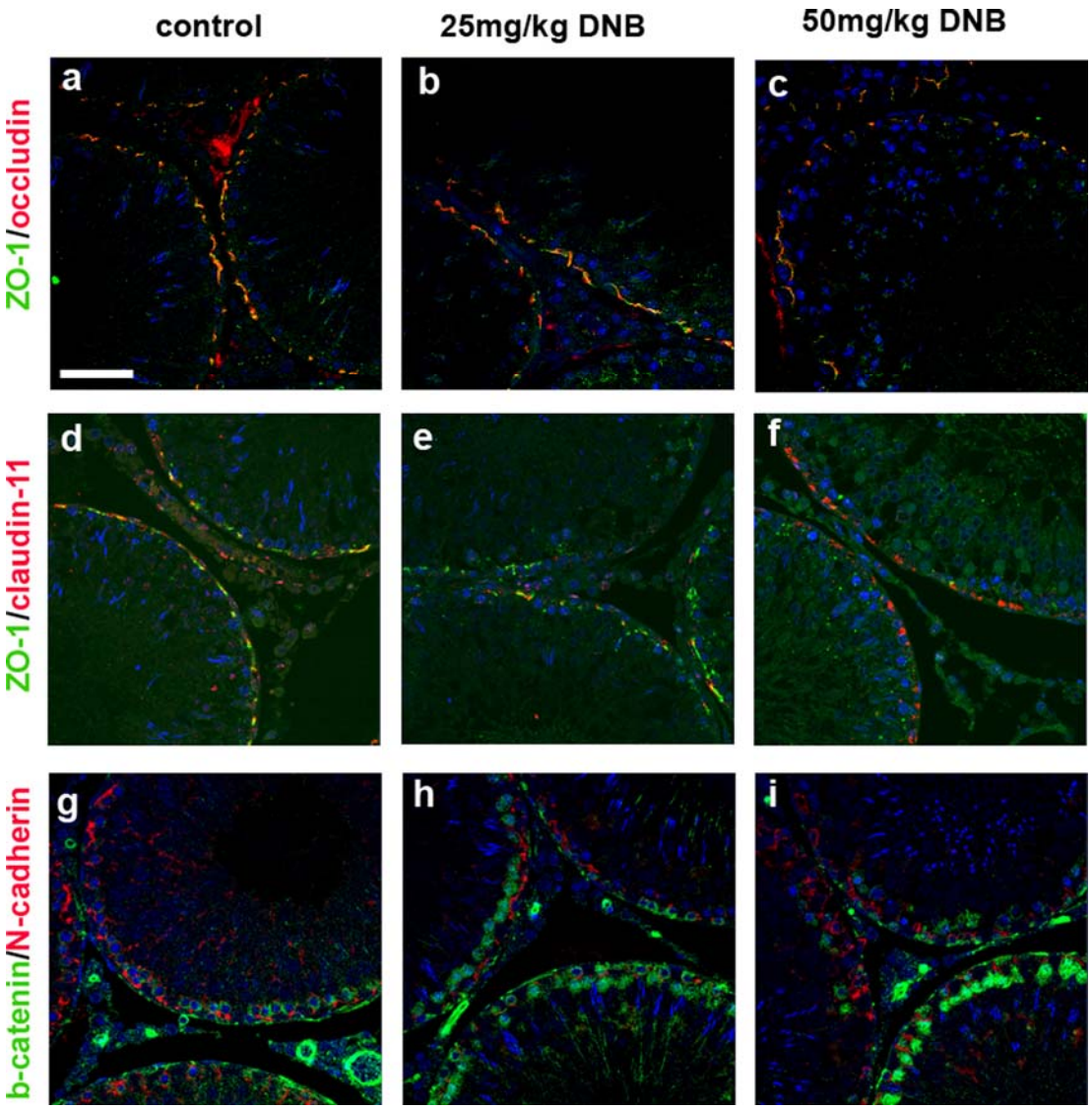


Figure 5-20 Testis sections from control (corn oil) rats and rats treated 24 hours earlier with 25mg/kg DNB or 50mg/kg DNB, co-immunofluorescence with a-c) ZO-1 (green) and occludin (red), d-f) ZO-1 (green) and claudin-11 (red) or g-i) β-catenin (green) and N-cadherin (red), all counterstained with dapi (blue). Scale bar represents 50μm.

Finally, the function of the barrier following DNB treatment was investigated using the biotin tracer. Figure 5-21 shows immunofluorescence detection of the biotin tracer, and the site of the BTB via detection of occludin. In control tissue (panel a), the biotin tracer is localised to the interstitum and the tubules, up to the site of the BTB. With tissue from animals treated with 25mg/kg or 50mg/kg DNB, the same distribution of biotin tracer can be seen (panels b, c), with no penetration of the biotin

tracer into the centre of the tubules. This shows that the blood-testis barrier is functioning, and agrees with the results described from Figure 5-20.

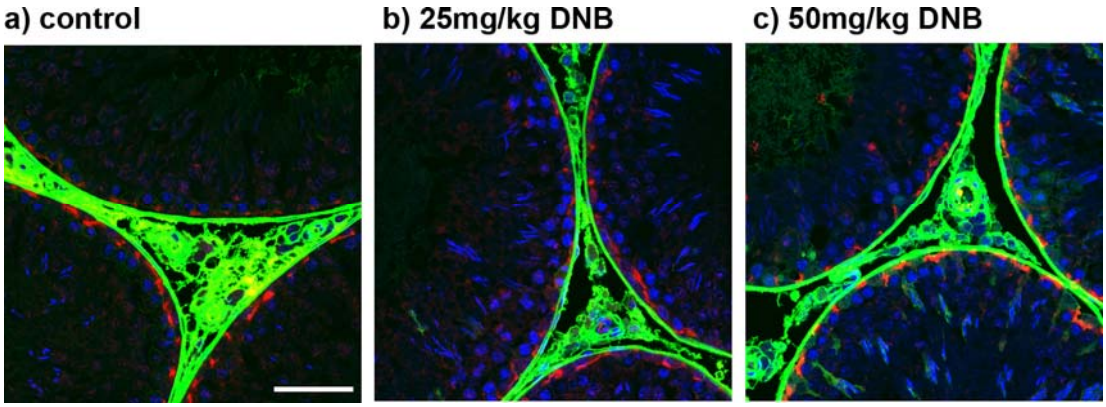


Figure 5-21 Detection of biotin tracer (green) and occludin (red) with Dapi nuclear counterstain, in testis sections from a) control (corn oil) rats and rats treated 24 hours earlier with b) 25mg/kg DNB, or c) 50mg/kg DNB. Scale bar represents 50µm.

Analysis of the genes for the BTB proteins, occludin, claudin-11, JAM-A, ZO-1, N-cadherin and β-catenin, by TaqMan® PCR is shown in Figure 5-22. The results showed no significant differences in expression of any of the genes investigated, relative to GAPDH expression, across the dose groups, suggesting that the DNB treatment has had no effect on expression of these genes in the 24 hours following administration of treatment. This agrees with the protein investigations above, where no differences in the BTB proteins were noted across the dose groups.

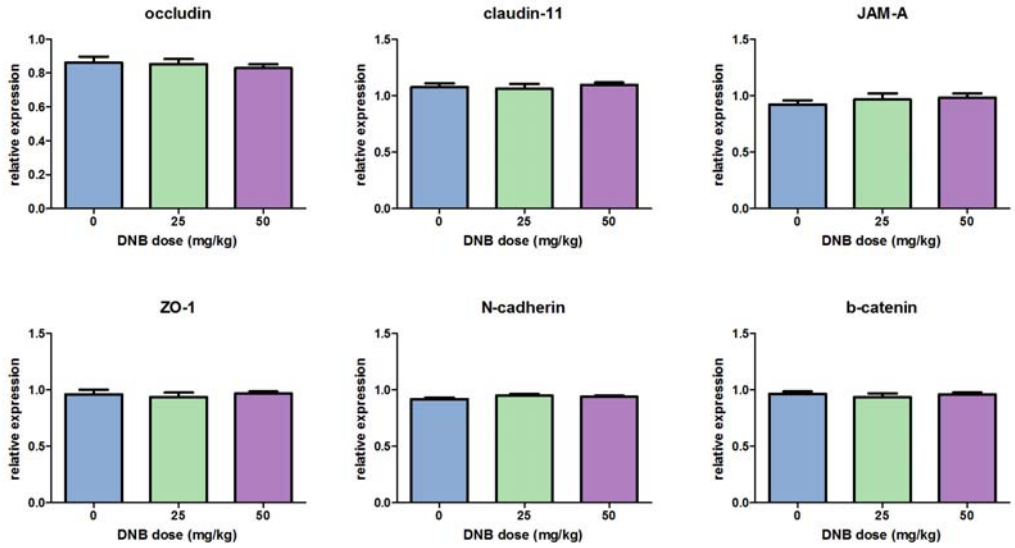


Figure 5-22 TaqMan® analysis of blood-testis barrier genes in testis samples collected from animals treated with DNB (mean with SEM). Expression relative to GAPDH expression. n=4 samples/dose group. No significant differences following statistical analysis (one-way ANOVA followed by Tukey post-test).

5.4 Discussion

It was hypothesised that germ cell proteins might leak from seminiferous tubules into interstitial fluid following toxicological insult. To investigate whether this protein leakage occurred and whether it was due to loss of integrity of the blood-testis barrier or was simply a consequence of germ cell damage, adult male rats were treated with one of three known testicular toxicants which target different aspects of the testis; cadmium chloride, methoxyacetic acid, 1,3-dinitrobenzene.

The damage caused by cadmium chloride treatment was investigated by immunohistochemistry with the general seminiferous tubule protein antibody. Dose dependent damage was observed, with disruption of the gross organisation of the seminiferous tubules following treatment with the intermediate (1.75mg/kg $CdCl_2$) and high (3mg/kg $CdCl_2$) doses. Observations at the sample collection stage also suggested an effect with the two higher doses. The testes from most of the animals in the 1.75 and 3mg/kg dose groups were a dark purple colour and were more turgid

than the control and low dose animals. Increases in interstitial fluid weight were also observed with the two higher dose groups. A 0.2mg/kg cadmium chloride dose group was included in initial studies but following histological examination, no differences were seen with this dose compared to vehicle control treatment. For this reason and considering the 3Rs, this dose was not included in further studies. The 1mg/kg, 1.75mg/kg and 3mg/kg groups were continued.

Leakage of proteins from the seminiferous tubules into interstitial fluid following high dose cadmium chloride treatment was suggested from Western blots using the STCM antibody. More protein bands were detected in IF samples from animals treated with 1.75mg/kg or 3mg/kg $CdCl_2$, and quantification of a 15kDa band suggested it was leaking from STs into IF. Quantification of a 32kDa protein band suggested that this protein was not leaking from STs into IF. This could be because this protein is naturally present in the interstitial fluid.

Protein leakage was further investigated using Coomassie dye stained gels, to evaluate changes in total protein in the IF samples. A clear dose related increase in the number of proteins present in the IF samples was identified across all four dose groups which suggested leakage of proteins from STs into IF following cadmium chloride treatment. Quantification of the protein bands suggested that some proteins increased in concentration in IF following treatment with the higher doses of $CdCl_2$, such as the 25kDa, and 15kDa bands. The concentration of some proteins in the IF samples did not appear to change with any dose of $CdCl_2$, such as the 45kDa protein; this could be because the protein is naturally present in IF, or perhaps more than one protein of the same size is present in the IF samples, masking any dose related changes. Other proteins appeared to be present in higher concentrations in the 1.75mg/kg dose group, compared to control, although no significant differences were observed between this dose group and the other two doses, suggesting that there could be a general effect on leakage of the proteins following treatment with any dose of cadmium chloride. Nevertheless, the Coomassie gels supported the Western blot results, and suggested that some proteins do leak out of seminiferous tubules

into interstitial fluid following treatment with high doses of cadmium chloride (1.75mg/kg, 3mg/kg).

The integrity of the blood-testis barrier was assessed to investigate whether the protein leakage observed could be due to loss of integrity of the barrier. Co-localisation studies of the tight junction proteins, ZO-1 and occludin, and ZO-1 and claudin-11, suggested that the tight junctions were disrupted following treatment with 1.75mg/kg or 3mg/kg cadmium chloride. Little or no ZO-1, occludin and claudin-11 protein was identified in the seminiferous tubules following these doses, with the little immunofluorescent staining that was evident suggesting that any protein present was not localised to the site of the blood-testis barrier. In comparison, the tight junction proteins were clearly co-localised at the site of the blood-testis barrier in control tissue and in that from animals treated with 0.2mg/kg and 1mg/kg cadmium chloride. The integrity of the adherens junctions in the BTB were also analysed using immunofluorescent co-localisation of N-cadherin and β-catenin. Whilst the results were generally in line with what was found for the tight junction proteins, the findings were less clear than with the tight junction proteins. N-cadherin was clearly localised at the site of the BTB in the control testes and in those from the 1mg/kg $CdCl_2$ dose group. In both groups, β-catenin was localised around the spermatocytes either side of the blood-testis barrier. Following treatment with either 1.75mg/kg or 3mg/kg $CdCl_2$, there appeared to be a reduction in the amount of both proteins present in the seminiferous tubules, and some disorganisation in the localisation of the proteins. This supports the tight junction results and confirms that the blood-testis barrier has probably been disrupted following treatment with high doses of cadmium chloride.

To definitively evaluate blood-testis barrier function, penetration of the biotin tracer was evaluated (Tarulli et al., 2008; Meng et al., 2005). The results showed localisation of the biotin tracer solely to the interstitum in control tissue and in testes from the 1mg/kg and 1.75mg/kg $CdCl_2$ dose groups, but with 3mg/kg cadmium chloride, the biotin tracer penetrated the seminiferous tubules, showing loss of

function of the blood-testis barrier. The results observed with the 1.75mg/kg dose group suggest that some seminiferous tubules could be affected by this intermediate dose, whereas some were unaffected.

Finally, the genes for some blood-testis barrier proteins were investigated using TaqMan®. The results were variable, and only a small number of samples were investigated. A significant decrease in expression of occludin was detected following treatment with 3mg/kg $CdCl_2$, agreeing with the protein data, which suggested that occludin was reduced in seminiferous tubules in animals treated with this dose. There were no statistically significant differences observed with the genes for the adherens junction proteins, N-cadherin and β-catenin, although a downward trend was observed with increasing $CdCl_2$ dose. A decrease in these proteins was suggested following the intermediate and high dose treatments but not a complete absence, which supports the trend in the mRNA expression data. With the other three tight junction genes, ZO-1, claudin-11 and JAM-A, a significant reduction in expression was noted following treatment with 1.75mg/kg cadmium chloride, compared to control. From the protein data, an effect on expression of these genes in the 3mg/kg dose group would have also been expected. The discrepancy could be due to the small number of samples used for the gene expression experiments, especially as one animal in the high dose group was excluded from the analysis due to the histology appearing normal, suggesting that the treatment had not worked.

The studies with cadmium chloride suggest that high doses (1.75mg/kg and 3mg/kg) cause disruption to the organisation of the seminiferous tubules, leading to leakage of some proteins from the tubules into the interstitial fluid. This protein leakage appears to be coincident with a loss of integrity of the blood-testis barrier.

The second testicular toxicant used was methoxyacetic acid. Two doses were selected to produce mild and moderate effects (200mg/kg and 650mg/kg). Immunohistochemical analysis of testis sections showed a dose dependent loss of

pachytene spermatocytes likely due to apoptosis of the cells. This supports published data on MAA toxicity (Bagchi and Waxman, 2008).

To investigate protein leakage following MAA treatment, Western blotting with the STCM antibody was carried out. No major differences were observed in the number of protein bands present in interstitial fluid samples from the control or either MAA dose group. Quantification of two protein bands (27kDa and 15kDa) hinted that there may have been an increase in the concentrations of the proteins following treatment with 650mg/kg MAA but there were no statistically significant differences. Analysis of total protein in IF samples was carried out using Coomassie dye stained gels. Again, no differences in protein concentrations in the IF samples from control and MAA treated animals were observed on the gels. Quantification of six protein bands (including those that appeared to leak out of STs in high dose cadmium chloride treated rats) supported this observation, and suggested that proteins are not leaking out of the seminiferous tubules into the interstitial fluid following treatment with MAA.

The integrity of the blood-testis barrier was evaluated using immunofluorescent co-localisation of the tight junction proteins, ZO-1 and occludin, and ZO-1 and claudin-11, and the adherens junction proteins, N-cadherin and β-catenin. The results showed the junctions in tissue collected from animals treated with either 200mg/kg MAA or 650mg/kg MAA were comparable to controls, which suggested that the blood-testis barrier was fully functional. This was confirmed directly using the biotin tracer. This showed that with both doses of MAA, the tracer was localised to the interstitum, as with control tissue. Similarly, expression analysis of blood-testis barrier genes showed no significant differences in testes from controls and from animals treated with either 200mg/kg or 650mg/kg MAA.

The third well-studied testicular toxicant used was 1,3-dinitrobenzene, due to its reported effects on Sertoli cells (Blackburn et al., 1988). Two doses were selected to cause mild and moderate effects (25mg/kg and 50mg/kg). Observations at sample

collection found one animal had died following treatment with the high dose, and other animals in the group looked unwell, suggesting that the treatment had caused severe adverse effects to the animals. Immunohistochemistry with the STCM antibody and DAZL antibody was used to assess the damage caused by the doses of DNB. The results showed that DNB caused Sertoli cell vacuolation and loss of spermatocytes with a more marked effect following treatment with 50mg/kg DNB, compared to 25mg/kg DNB, as expected from the published literature. Immunohistochemistry with TUNEL suggested that the loss of germ cells was due to apoptosis.

Protein leakage from seminiferous tubules into interstitial fluid following DNB treatment was evaluated by Western blot analysis with the STCM antibody. The results suggested that there were no differences in the proteins detected in control interstitial fluid samples, and those from animals treated with either dose of DNB, although there appeared to be an error in the amount of protein loaded into one lane of one gel (Figure 5-18). Quantification of two protein bands (27kDa and 15kDa) suggested no statistically significant differences in protein concentration between IF samples from control or DNB treated groups. Analysis of total protein using Coomassie stained gels, supported the Western blot results. No differences in the protein bands between dose groups were observed on the gels, and quantification of six protein bands showed no statistically significant differences between the groups.

Immunofluorescence was again used to investigate the co-localisation of the tight junction proteins, ZO-1 and occludin, and ZO-1 and claudin-11, and the adherens junction proteins, N-cadherin and β-catenin to investigate the integrity of the blood-testis barrier. The results suggested that there were no differences in the localisation and presence of these proteins in tissue from animals treated with either 25mg/kg or 50mg/kg DNB and control tissue. Analysis of blood-testis barrier function with the biotin tracer showed the tracer localised to the interstitum in testes from both treatment groups and controls. These results suggest that treatment with either 25mg/kg or 50mg/kg DNB has not disrupted the blood-testis barrier. Further support

for this conclusion came from analysis of expression of the genes for occludin, claudin-11, JAM-A, ZO-1, N-cadherin and β-catenin, which showed no differences in expression between tissue collected from animals treated with DNB or vehicle.

The results presented in this chapter suggest that damage to the pachytene spermatocytes caused by MAA treatment, and damage to the Sertoli cells, caused by DNB treatment, do not cause disruption of the blood-testis barrier, and do not lead to leakage of proteins from the seminiferous tubules into the interstitial fluid. Therefore, induction of germ cell damage/degeneration, whether directly (MAA) or via perturbation of Sertoli cells (DNB), is not sufficient on its own to cause leakage of germ cell specific proteins into IF. Treatment with high doses of cadmium chloride caused disruption to the gross organisation of the seminiferous tubules, including germ cell loss/degeneration and also induced loss of integrity of the blood-testis barrier. Leakage of proteins from the tubules into IF was observed following high dose cadmium chloride treatment. This suggests that loss of integrity of the blood-testis barrier is a pre-requisite for protein leakage from the STs into IF.

To follow up on this conclusion, treatments to specifically target the blood-testis barrier will be investigated in chapter 6.

6 Is Loss of Integrity of the Blood-Testis Barrier required for Protein Leakage from Seminiferous Tubules into Interstitial Fluid?

6.1 Introduction

The results presented in the previous chapter suggested that potential biomarker proteins will only leak out of seminiferous tubules (STs) into interstitial fluid (IF) following a loss of integrity of the blood-testis barrier (BTB). This has implications for the potential use of biomarkers for testicular toxicity as it suggests that a sensitive biomarker for general testicular toxicant-induced damage may not be possible to identify.

In order to confirm this hypothesis, three approaches were taken to specifically target the BTB and investigate whether this really is integral to the problem.

6.1.1 Development of the Blood-Testis Barrier

The first approach used to try and confirm the hypothesis involved study of the development of the blood-testis barrier. The testes begin to develop early during embryogenesis (around day 12.5-13.5 of gestation in the rat, (Magre and Jost, 1991)) and continue to develop into adulthood. Spermatogenesis begins at puberty, following the formation of the BTB. In the rat, the onset of spermatogenesis occurs around postnatal day 8 (Vitale et al., 1973) with the formation of the blood-testis barrier occurring around postnatal days 15-19 (Vitale et al., 1973; Russell et al., 1989). This can be used to look at the leakage of proteins from STs into IF from a different point of view. In theory, germ cell proteins should be present in the interstitial fluid before the BTB is formed, but following BTB formation, they should not be identified in IF due to the presence of the barrier preventing them leaking out.

The development of the blood-testis barrier was studied at 4 ages; postnatal day 10, 15, 18 and 25, and the proteins in IF samples analysed in order to investigate this idea.

6.1.2 Glycerol

The second approach to further investigate the role of the BTB and protein leakage from STs involved administration of glycerol. Intra-testicular injection of glycerol was first shown to inhibit spermatogenesis in rats by Wiebe and Barr, 1984. They noted a depletion of spermatogenic cells, within 2 weeks of treatment but no effect on steroidogenesis (Wiebe and Barr, 1984). Eng et al., 1994, investigated the mechanism by which glycerol may exert its effects and suggested that a single intra-testicular injection of a low concentration of glycerol can cause permanent damage to the seminiferous tubules by altering the permeability of the BTB. The effect of glycerol treatment on the BTB was further characterised by Wiebe et al., 2000, who found that glycerol caused specific disruption to the organisation of the Sertoli cell cytoskeleton (actin filaments and microtubules) and the tight junction protein, occludin, however the mechanism or initial target of glycerol was not investigated.

Intra-testicular injections of glycerol were used to specifically target the BTB to evaluate whether this leads to leakage of proteins from STs.

6.1.3 Transforming Growth Factor-β3

Treatment with transforming growth factor-β3 (TGF-β3) was the third approach used to investigate the role of the BTB in leakage of proteins from STs into IF. Xia et al., 2006, reported that overexpression of TGF-β3 in primary cultured Sertoli cells led to disruption of tight junctions and adherens junctions. They went on to suggest an in vivo model for studying BTB dynamics by intra-testicular injection of TGF-β3. They showed that local administration of TGF-β3 caused reversible disruption to the tight junctions and basal ectoplasmic specialisations at the BTB and to the apical

ectoplasmic specialisations between spermatids and Sertoli cells (Xia et al., 2006). Further analysis showed specific reductions in the levels of occludin, ZO-1, N-cadherin and nectin-3 protein levels, but no change in JAM-A or β-catenin protein levels following administration of TGF-β3 (Xia et al., 2006). Xia et al., 2008, further validated this model system and suggested that TGF-β3 may be causing disruption to the BTB by clathrin-mediated endocytosis of the BTB proteins.

Local administration of TGF-β3 was used as the third approach to pinpoint whether disruption to the BTB and more specifically to the tight junctions is required for leakage of proteins from STs.

6.1.4 General Aims

The aim of the studies in this chapter is to further investigate the results observed in chapter 5 and to evaluate whether loss of integrity of the blood-testis barrier is required for leakage of proteins from seminiferous tubules into interstitial fluid. Three approaches were taken. Firstly testis development was used as a physiological approach to investigate whether there are changes in the amounts of proteins present in IF before and after the BTB forms. Secondly, two experimental approaches to specifically target the BTB were used, intra-testicular injection of glycerol, and intra-testicular injection of TGF-β3.

As in chapter 5, the damage caused by the treatments was investigated by immunohistochemistry, assessment of the blood-testis barrier using immunofluorescent techniques, and the interstitial fluid protein content evaluated by Western blotting and Coomassie gel analysis.

6.2 Methods

6.2.1 Development of the Blood-Testis Barrier

Samples were collected from male Wistar rats at four different ages, postnatal days 10, 15, 18 and 25, for comparison with control adult tissue. These ages were selected to encompass the period in which the blood-testis barrier develops. Testes were collected and snap-frozen for PCR (section 3.4.2.2), or injected with biotin tracer (section 3.3.6). Interstitial fluid samples were also collected. Please refer to section 3.4 for sample collection details. Fixed tissue collected previously in the lab was used for immunohistochemical analyses. For fixed tissue, interstitial fluid samples, and frozen tissue, at least two testes were used per age, depending on availability of tissue, and success of the sample collection. For biotin tracer injected tissue, one animal was used at each age.

6.2.2 Glycerol Treatment

Adult male rats were treated with glycerol by intra-testicular injection as described in section 3.3.4. An initial investigation collecting testicular tissue 24 hours after treatment was carried out but the treatment appeared to have no effect so a second study was set up to collect tissue 48 hours after administration of the treatment.

Testes were collected for fixation, injection of biotin tracer, and interstitial fluid collection. The number of samples collected is detailed in Table 6-1. No obvious effects were observed during administration of the treatment or sample collection.

Table 6-1 Number of Samples collected from glycerol study. For fixed tissue (n) indicates number of fixed tissue samples injected with biotin tracer.

Treatment	Number of Samples	
	Fixed Tissue	Interstitial Fluid
15% glycerol (right testis)	8 (4)	4
Saline (left testis)	8 (4)	4

6.2.3 TGF-β3 Treatment

Adult male rats were treated with TGF-β3 by intra-testicular injection as described in section 3.3.5. An initial investigation collecting testicular tissue 24 hours after treatment was carried out but the treatment appeared to have no effect so a second study was set up to collect tissue 48 hours after administration of the treatment.

Testes were collected for fixation, injection of biotin tracer, and interstitial fluid collection. The number of samples collected is detailed in Table 6-2.

During administration of the TGF-β3 treatment, one animal appeared to have much smaller testes than the others. Following sample collection, and analysis of the fixed tissue, the testes from this animal were not fully developed, so were not analysed further.

Table 6-2 Number of Samples collected from TGF-β3 study. For fixed tissue (n) indicates number of fixed tissue samples injected with biotin tracer.

Treatment	Number of Samples	
	Fixed Tissue	**Interstitial Fluid**
200ng TGF-β3 (right testis)	6 (3)	3
PBS (left testis)	6 (3)	3

6.2.4 Immunohistochemistry and Immunofluorescence

Fixed testes were sectioned as described in section 3.4.2.1.1. Immunohistochemistry was carried out with the STCM antibody and an antibody to DAZL. Immunofluorescence was carried out to analyse distribution of the biotin tracer, and co-localisation of ZO-1 and occludin, and N-cadherin and β-catenin. Detailed methods are described in chapter 3.

6.2.5 1D Gel Analysis and Western Blotting

Proteins present in interstitial fluid samples were analysed by running samples on 1D gels, then staining with GelCode Coomassie based stain, or Western blotting with the STCM antibody or an antibody to DAZL (samples from the development study). Details of these methods are included in chapter 3.

6.2.6 TaqMan® Q-PCR

RNA was extracted from snap-frozen tissue from the development study. cDNA was made and used for TaqMan® Q-PCR analysis of the blood-testis barrier genes, occludin, claudin-11, JAM-A, ZO-1, N-cadherin, and β-catenin, and the control gene, GAPDH. Detailed methods are described in chapter 3, along with primer and probe sequences used for the TaqMan® reaction.

6.3 Results

6.3.1 Development of the Testis and Blood-Testis Barrier

The first approach involved analysis of proteins in interstitial fluid samples before, during, and after blood-testis barrier formation.

6.3.1.1 Blood-Testis Barrier Formation

The formation of the blood-testis barrier (BTB) during the development of the testis was confirmed by immunofluorescence for the tight junction proteins, occludin and ZO-1, and the adherens junction proteins, N-cadherin and β-catenin, as well as functional analysis using the biotin tracer, and TaqMan® analysis of the BTB genes. Figure 6-1 shows the localisation of ZO-1 and occludin at 5 different ages. Co-localisation of both proteins was evident at day 18, although some tubules appeared less developed than others. By day 25, all tubules showed co-localisation of ZO-1 and occludin at the site of the BTB, comparable to the staining seen in adult tissue. Between day 18 and day 25, it appears that the tight junction proteins are moving out

from the centre of the tubules towards the basement membrane as the tubules develop, until they reach the final site of the BTB. At day 10 and day 15, no occludin protein was detected in the seminiferous tubules. Some ZO-1 staining was identified at both ages, in a network pattern around the germ cells. This suggests that ZO-1 has a role in early development of the testis, before its role in the BTB. These results suggest that the tight junctions in the BTB are beginning to form between days 15-18, and are fully formed by day 25, in agreement with the literature.

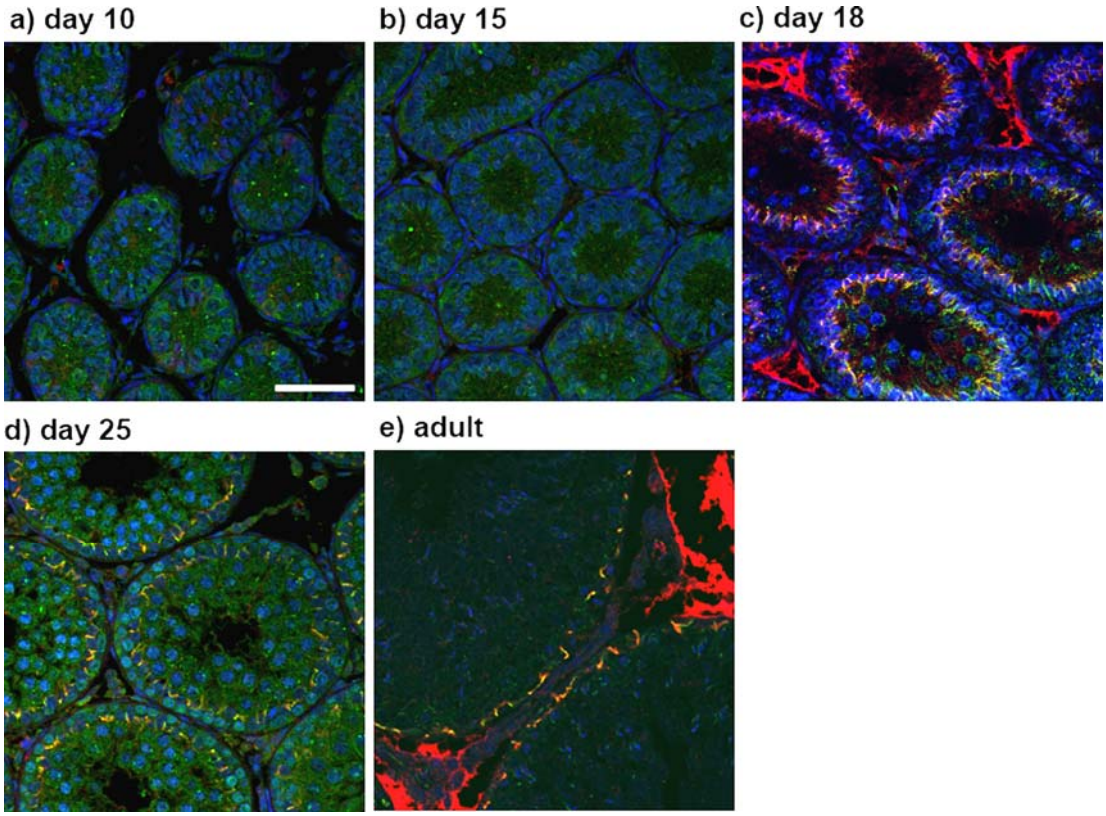


Figure 6-1 Co-localisation of ZO-1 (green) and occludin (red) with Dapi nuclear counterstain (blue), on testis sections from rats at a) day 10, b) day 15, c) day 18, d) day 25, and e) adulthood. Scale bar represents 50µm.

The development of the adherens junctions was also investigated using immunofluorescent detection of N-cadherin and β-catenin. Figure 6-2 shows the presence of N-cadherin (red staining) throughout the development of the testis but with a change in localisation. At day 10 and 15, N-cadherin appeared to be centrally located in the tubules, in a network pattern around the developing germ cells. By day

18, the protein began to localise towards the basement membrane of the tubules, and at day 25, it was present at the site of the BTB, comparable to adult tissue. β-catenin exhibited a different pattern during development of the testis. There was no β-catenin present at day 10, although it appeared to be present at day 15 in the interstitum, and around some of the germ cells. At day 18, when the N-cadherin was localising to the site of the BTB, the pattern of β-catenin protein did not seem to change. At day 25, the same pattern was clearer with β-catenin localised to spermatocytes and interstitial components, comparable to the β-catenin staining seen in adult tissue.

These results suggest that N-cadherin expression follows the same pattern seen with the tight junction proteins, ZO-1 and occludin, during development of the testis and formation of the BTB. On the other hand, β-catenin exhibited the same staining pattern throughout the development process, suggesting it has a different role.

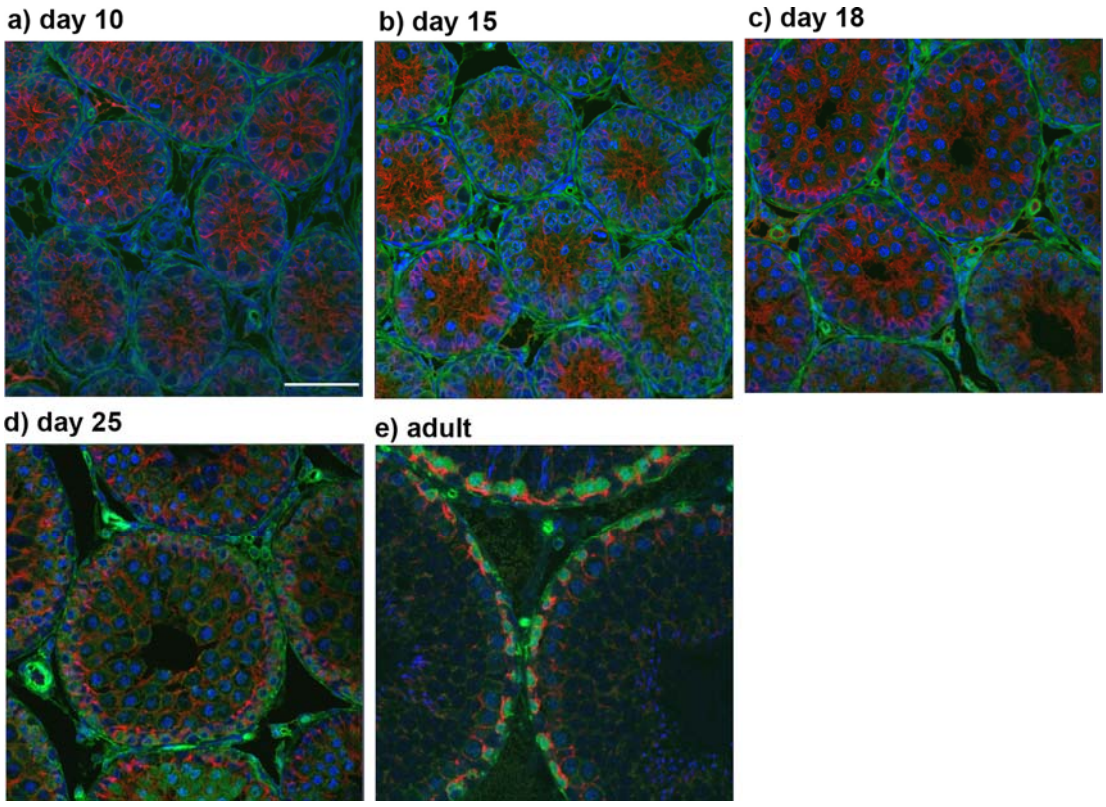


Figure 6-2 Co-localisation of β-catenin (green) and N-cadherin (red) with Dapi nuclear counterstain (blue), on testis sections from rats at a) day 10, b) day 15, c) day 18, d) day 25 and e) adulthood. Scale bar represents 50μm.

The function of the BTB during development of the testis was investigated using the biotin tracer. The tracer should be located in the interstitum and around the germ cells up to the site of the BTB if the barrier is functional. Following loss of integrity of the BTB, the biotin tracer can penetrate the seminiferous tubules. Therefore, it was expected that the tracer would be found throughout the tubules prior to formation of the BTB. Figure 6-3 shows the biotin tracer at different ages of testis development. Occludin is also shown to indicate the presence of the BTB from day 18 onwards. With day 18 and day 25 tissue the biotin tracer was localised to the interstitum and surrounding the germ cells up to the site of the BTB, which is comparable to adult tissue and indicates that the BTB is functional from day 18. The same pattern was, however, also seen with day 10 and day 15 testes. There was a slight difference with the day 15 tissue in which some biotin staining was seen in the centre of the tubules. This is similar to some tubules in adult tissue, described in chapter 4, and does not look like penetration of the tracer throughout the tubules. This result is unexpected and suggests that some sort of barrier is in place before the full BTB is formed. Perhaps some other BTB proteins are present, or the basement membrane has a barrier function at this stage in testis formation to protect the developing germ cells.

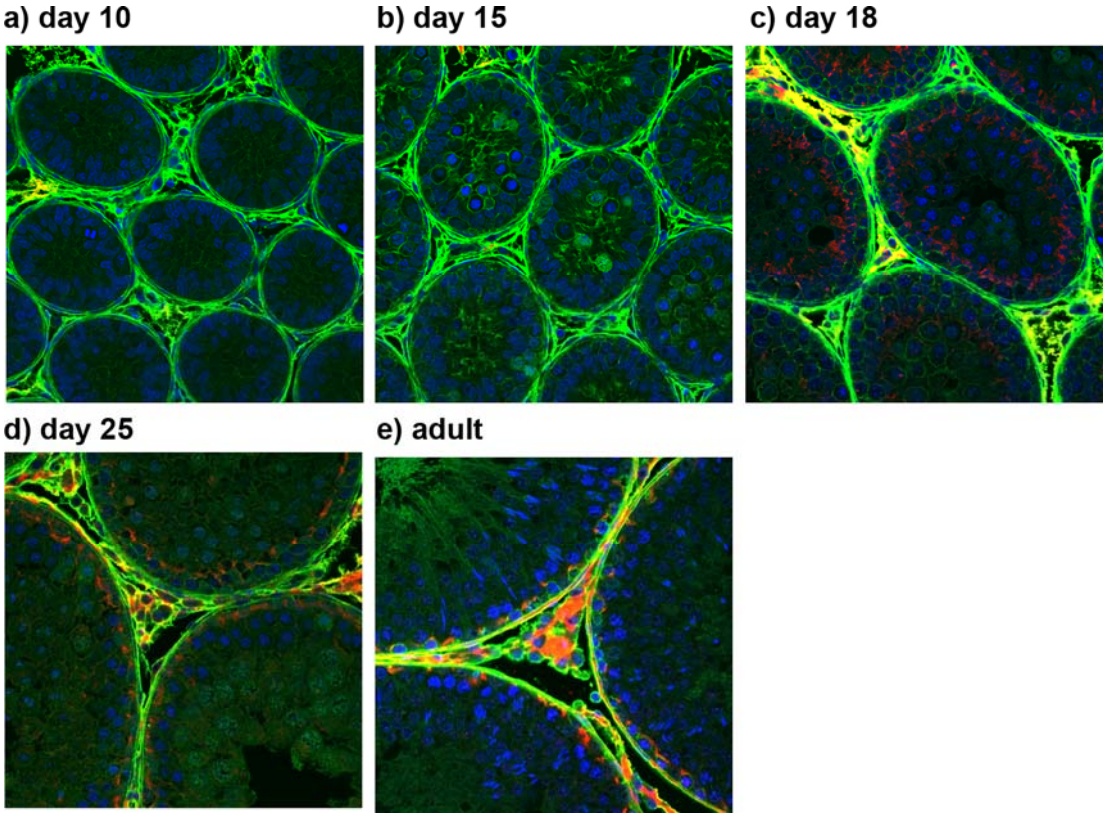


Figure 6-3 Detection of biotin tracer (green) and occludin (red) with Dapi nuclear counterstain (blue), on testis sections from rats at a) day 10, b) day 15, c) day 18, d) day 25, and e) adulthood. Scale bar represents 50μm.

Analysis of the genes for the tight junction proteins, occludin, claudin-11, JAM-A, and ZO-1, and adherens junction proteins, N-cadherin and β-catenin are shown in Figure 6-4. The results showed generally steady expression of all six genes across the age range. It is important to note that a lot of variability was seen in the individual data for each sample, and only a small number of samples were used suggesting that these results may not be very reliable. The results suggested statistically significant increases in expression of occludin, claudin-11 and ZO-1 at day 25 in comparison to adult. Significantly increased occludin mRNA expression was also observed at day 10 and day 18 compared to adult, whereas the protein data suggested that occludin protein was not present until day 18. No differences in mRNA expression of N-cadherin and β-catenin (except an increase in β-catenin expression at day 10) were noted across the age range, which does perhaps agree with the protein data shown in

Figure 6-2, which all appear to be located in the tubules throughout testis development although their location in the tubules changes.

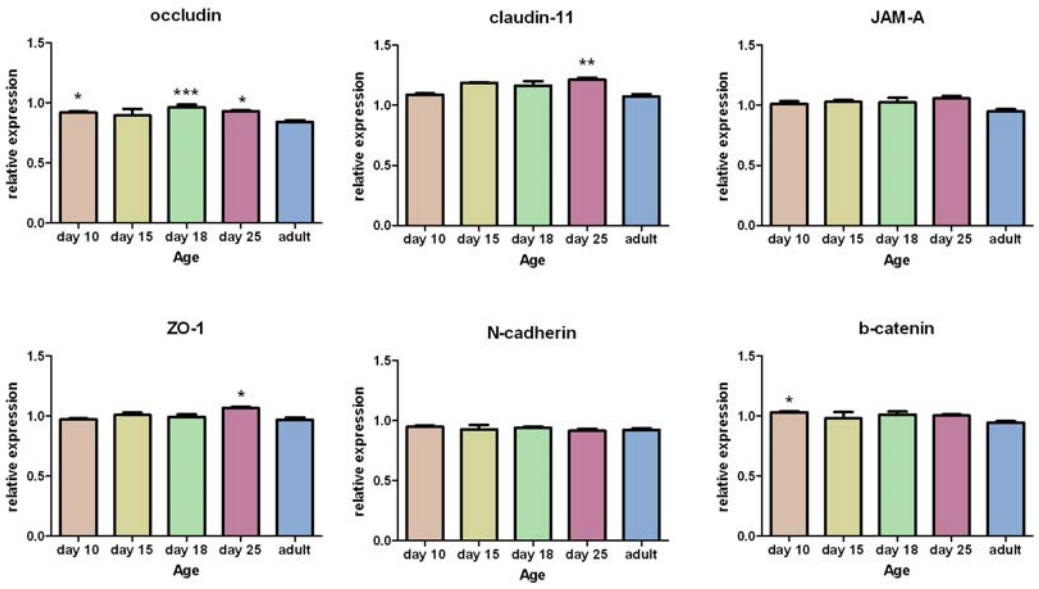


Figure 6-4 TaqMan® analysis of blood-testis barrier genes in testis samples collected from day 10, day 15, day 18, day 25 and control adult rats (mean with SEM). Expression relative to GAPDH expression. n=4 samples/age except day 15 where n=2 and adult where n=12. Statistical analysis, one-way ANOVA followed by Tukey post-test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to adult samples.

6.3.1.2 Protein Analysis of Interstitial Fluid Samples

Analysis of proteins present in interstitial fluid samples and isolated seminiferous tubules was carried out using Western blotting with an antibody raised to seminiferous tubule conditioned medium (STCM). Before samples were analysed, immunohistochemistry was carried out on tissue sections from day 10, 15, 18, 25, and adult rats with the STCM antibody to see if the STCM antibodies recognised proteins expressed at these ages (the antibody detects a range of different sized proteins of an unknown nature in adult samples). Figure 6-5 shows testis sections from day 10, 15, 18, 25 and adult rats immunostained with the STCM antibody. The antibody detected proteins present in seminiferous tubules at each age, although not all the proteins detected in the adult tissue appeared to be present through the age

range. Nevertheless, the results suggest that the STCM antibody can still be used to investigate proteins present in IF and ST samples.

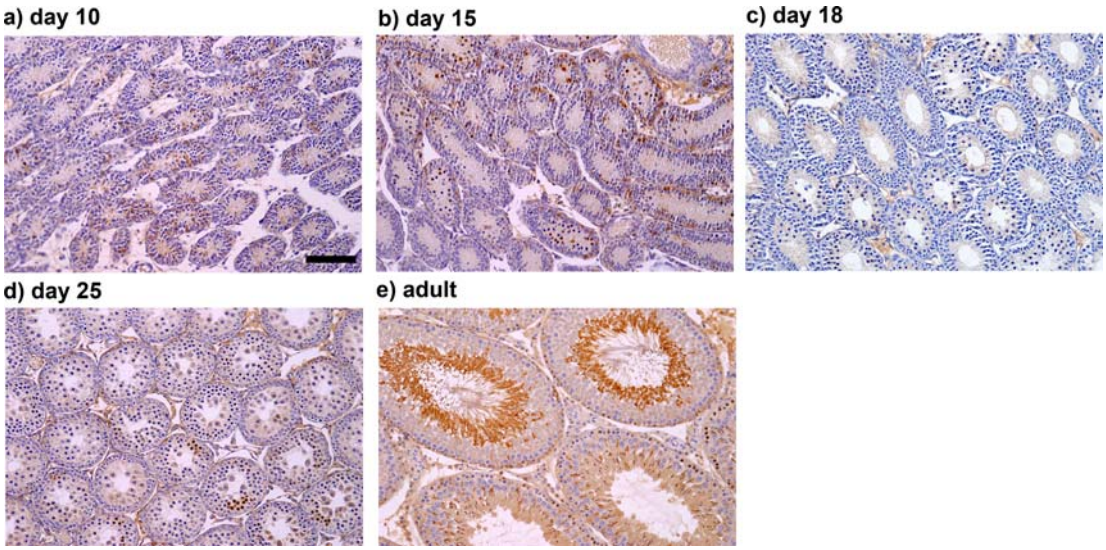


Figure 6-5 Testis sections from rats at a) day 10, b) day 15, c) day 18, d) day 25, and e) adulthood, immunostained with an antibody to seminiferous tubule conditioned medium (brown) and counterstained with haematoxylin (blue). Scale bar represents 100μm.

Interstitial fluid samples collected from rats across the age range, were run on a Western blot with the STCM antibody, to investigate the hypothesis that germ cell derived proteins will be present in IF samples before the BTB is formed but not afterwards. The blot is shown in Figure 6-6 with quantification of some protein bands of interest. The Western blot showed that different proteins are present in IF at different stages of testis development. Because the proteins the antibody detects are unknown, it is hard to select germ cell proteins from the blot for quantification analysis. Looking at tissue samples from such young testes also has the added problem that many germ cell proteins will not be present until later in development, or even until the adult stage. Different patterns can be seen with the proteins chosen for quantification from the Western blot. The 45kDa protein showed a decrease in the amount present in IF at day 18, with a large amount present at day 10. With the 39kDa and 28kDa proteins, there was variability across the age range. The 15kDa protein showed very little or absence at days 10, 15, and 18, with a small amount

present in IF at day 25 and a much larger amount in the adult IF. This result could be because the protein is not expressed until later germ cells emerge, rather than an increase in leakage of the protein from the STs across the age range.

Seminiferous tubules were isolated from testes at the different ages to try and identify the origin of some of the proteins seen in the IF samples. Isolated STs were run on a Western blot with the STCM antibody. Figure 6-7 shows that the proteins present in the ST samples, identified with this antibody were fairly consistent across the age range, with more proteins present in the adult sample. One of the proteins quantified in the previous figure, 15kDa, was again quantified because a clear band was visible in the day 25 sample, as well as in the adult sample. This result suggests that this protein is expressed in the later stages of spermatogenesis, because it is present in STs but only following formation of the BTB and the start of spermatogenesis.

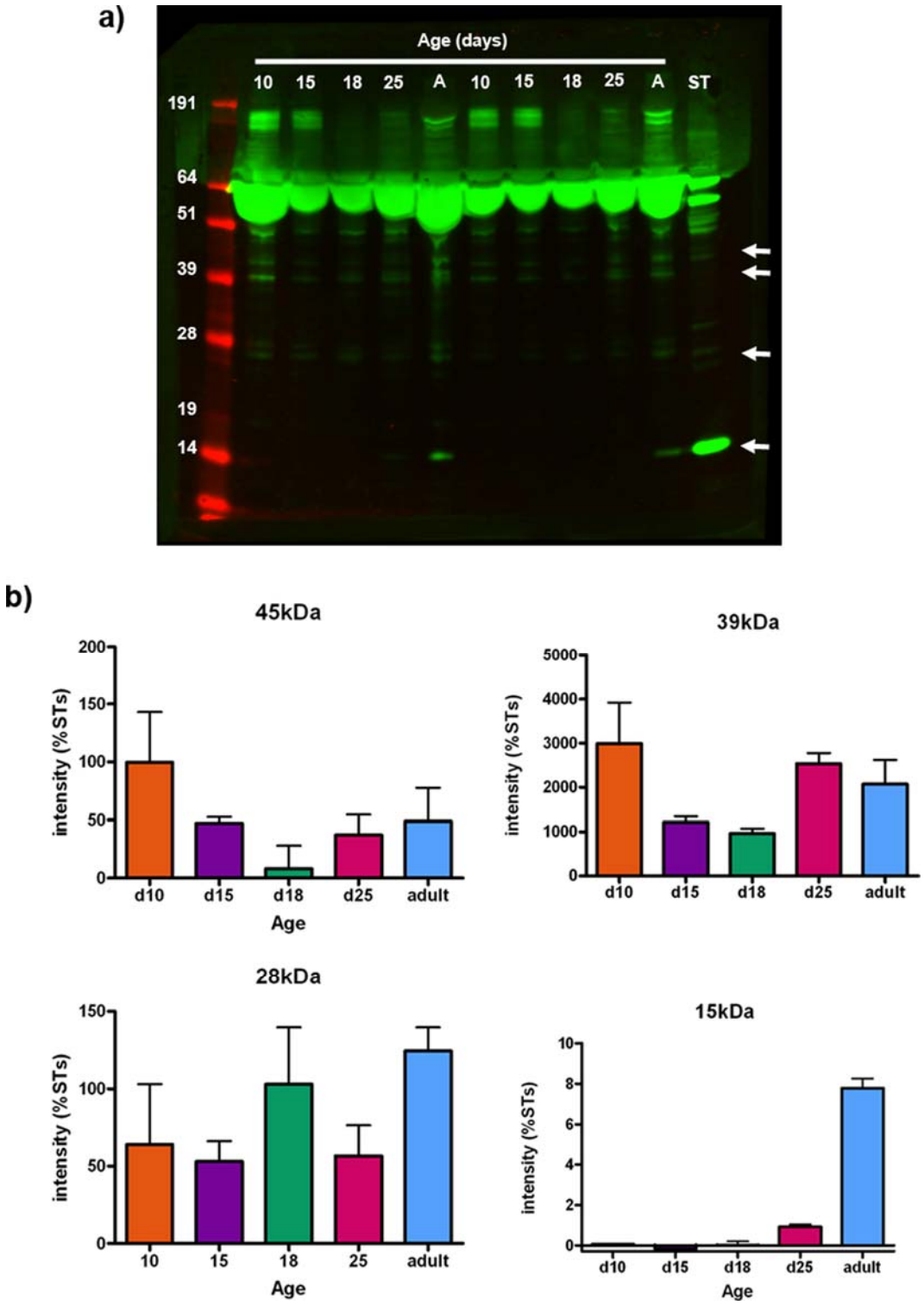


Figure 6-6 a) Western Blot with antibody to STCM, showing interstitial fluid samples from day 10, 15, 18, 25 and adult (A) rats and control homogenised seminiferous tubules (ST). Arrows indicate examples of protein bands quantified. b) Graphs showing quantification of indicated bands (mean with SEM), with values taken from samples run on one blot, expressed as a percentage of STs (n=2 samples/age).

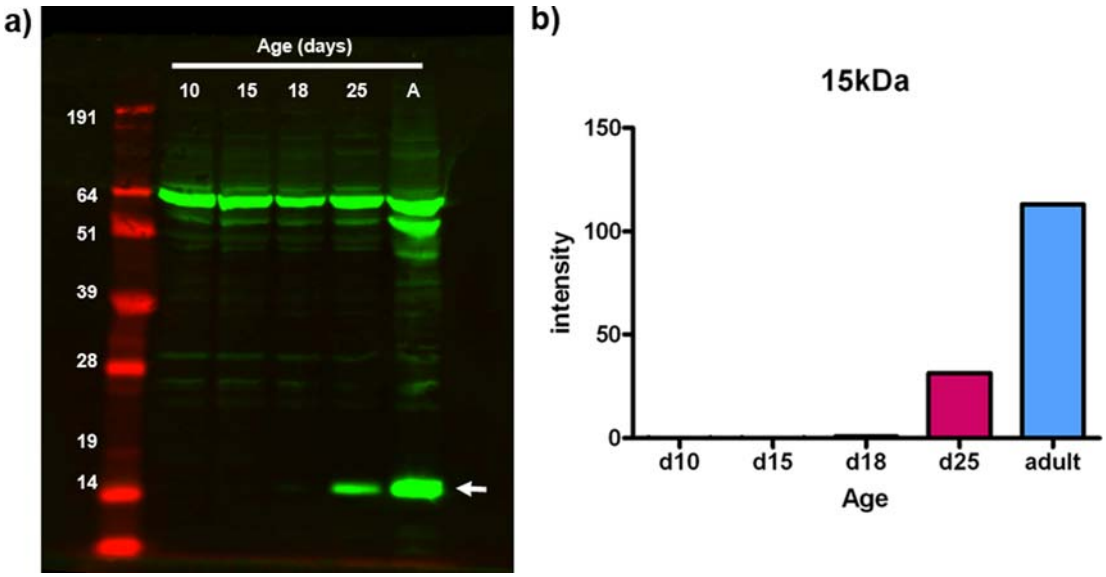


Figure 6-7 a) Western Blot with antibody to STCM, showing seminiferous tubule samples from day 10, 15, 18, 25 and adult (A) rats. Arrow indicates protein band quantified. b) Graph showing quantification of 15kDa bands (mean with SEM) (n=1 sample/age).

As with protein analysis in IF samples in chapter 5, IF samples from the different aged rats were run on Coomassie stained gels to evaluate total protein content rather than just those proteins detected with the STCM antibody. The gel and quantification of some protein bands are shown in Figure 6-8. Many more proteins were detected with this method than with Western blotting with the STCM antibody, and some variation across the age range was evident. From the quantification, proteins of 191kDa and 39kDa, in size, showed similar patterns with an increase in the amount of protein in adult IF samples, suggesting that they could be expressed in the later stages of spermatogenesis, and could be expressed in Sertoli cells rather than germ cells. The 28kDa protein had a similar pattern, but with an increase at day 25 as well as in adult samples, again, suggesting that it is likely to be a product of the Sertoli cells switched on as the testis developed. The proteins of 45kDa, and 27kDa in size, did not appear to change much across the age range suggesting that they are interstitial compartment proteins, or present in the basal tubule compartment, or secreted from the seminiferous tubules. Unlike in the Western blot in Figure 6-6, the 15kDa appeared to be very variable in the amount present in IF samples across the age range.

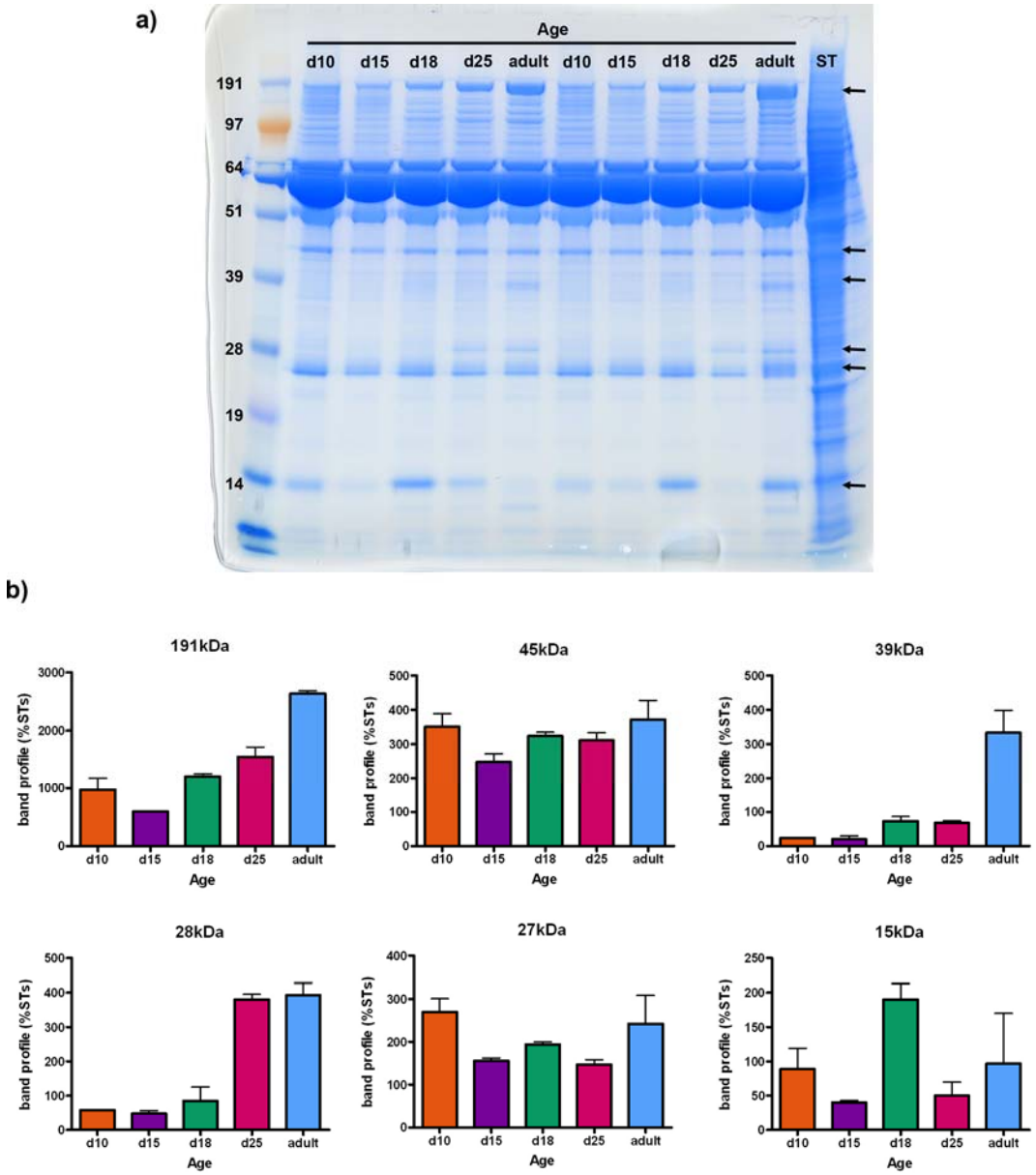


Figure 6-8 a) Coomassie gel showing interstitial fluid samples from day 10, 15, 18, 25 and adult (A) rats and control homogenised seminiferous tubules (ST). Arrows indicate examples of protein bands quantified. b) Graphs showing quantification of indicated bands (mean with SEM), with values taken from samples run on one gel, expressed as a percentage of STs (n=2 samples/age).

6.3.1.3 Analysis of Germ Cell Specific Proteins in IF Samples

The general approach to investigating proteins present in IF samples from animals during testis development to investigate the hypothesis that germ cell derived proteins should leak into IF before the BTB has formed could be unsuccessful because the different stages of germ cells are not present at such young ages, and spermatogenesis only begins at day 8 in the rat with formation of the BTB at day 15-19. One germ cell protein that is expressed from day 10 is DAZL. DAZL protein, along with the germ cell proteins, calpastatin and VASA were investigated by immunohistochemistry and Western blotting (DAZL and VASA). Calpastatin is expressed in spermatids, and was only expressed in adult tissue (data not shown) so no differences in IF would be detected across the age range. VASA was expressed in spermatocytes from day 10, but was not identified in IF samples (data not shown). Immunohistochemistry with DAZL across the age range is shown in Figure 6-9, and DAZL is clearly present in germ cells from day 10.

IF samples from the different aged rats were run on a Western blot with an antibody to DAZL. Results and the quantification of the band at about 25kDa are shown in Figure 6-10. Although DAZL protein is described as being 33kDa, the bands identified in the IF samples on the Western blot do appear to be DAZL protein. A band about 39kDa in size was identified in the control adult ST sample. Perhaps the protein is processed and shortened when leaking out of the seminiferous tubules, resulting in a protein about 25kDa in length. The blot suggested that there are no differences in the amount of DAZL present in IF samples taken from day 10, 15, 18, 25 or adult rats.

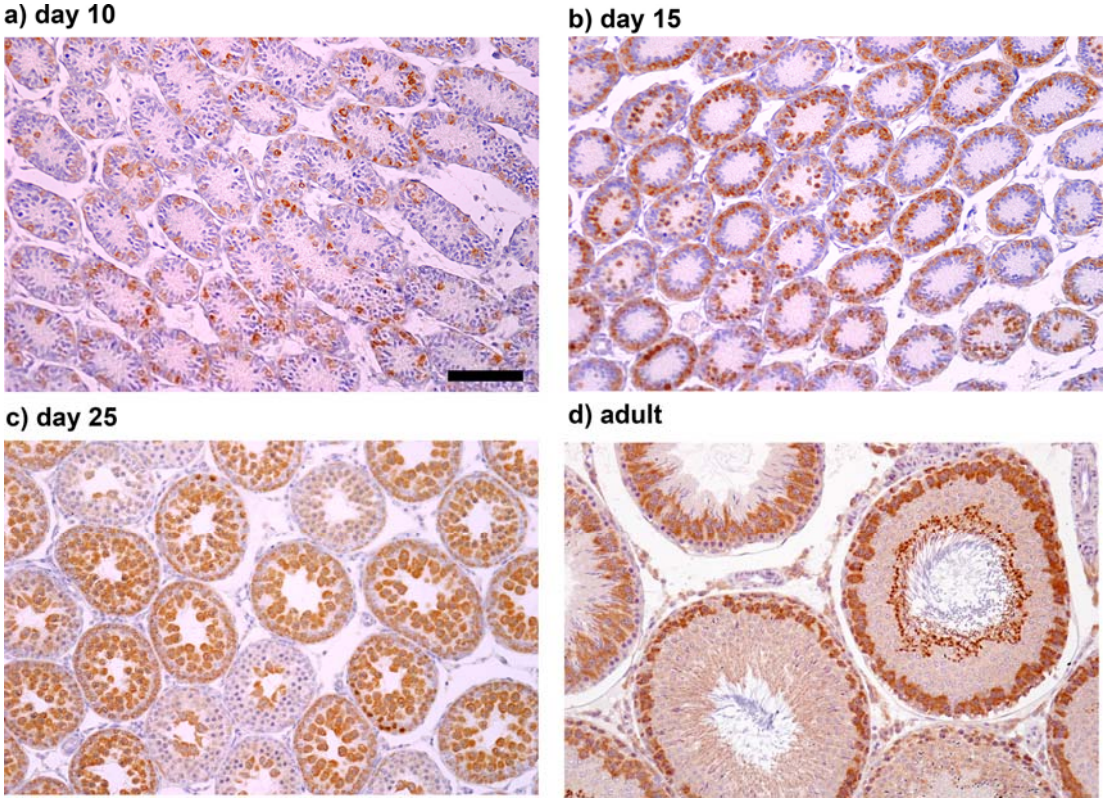


Figure 6-9 Testis sections from rats at a) day 10, b) day 15, c) day 25, and d) adulthood, immunostained with an antibody to DAZL (brown) and counterstained with haematoxylin (blue). Scale bar represents 100µm.

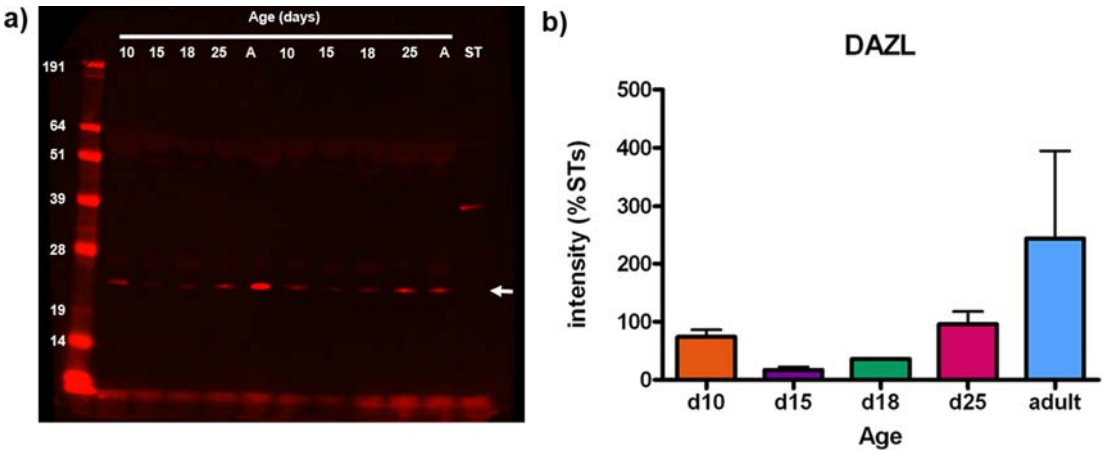


Figure 6-10 a) Western Blot with antibody to DAZL, showing interstitial fluid samples from day 10, 15, 18, 25 and adult (A) rats and control homogenised seminiferous tubules (ST). Arrow indicates protein bands quantified. b) Graphs showing quantification of 25kDa band, thought to be DAZL (mean with SEM), with values taken from samples run on one Western blot, expressed as a percentage of STs (39kDa band used for normalisation) (n=2 samples/age).

6.3.2 Glycerol Treatment

6.3.2.1 Analysis of Damage caused by Glycerol Treatment

Testis sections from rats treated with glycerol were immunostained with the general antibody to seminiferous tubule proteins (STCM) to analyse any damage caused to the structure of the seminiferous tubules. A section from a control testis and three sections from the concurrent glycerol treated testis are shown in Figure 6-11. The panels show the range in affect across the section of the treated testis. Panel d is comparable to the control section shown in panel a, but damage can be seen to the tubules shown in panels b and c. In b, there seems to have been a loss of germ cells from the tubules, and in c, vacuolation and loss of organisation of the seminiferous epithelium. As shown by the variation of damage across the section, the effect of the glycerol was localised with much of the tissue section looking normal. It is assumed that the damage is localised to the area the glycerol was injected into, although it was hard to confirm the site of injection in the paraffin embedded tissue.

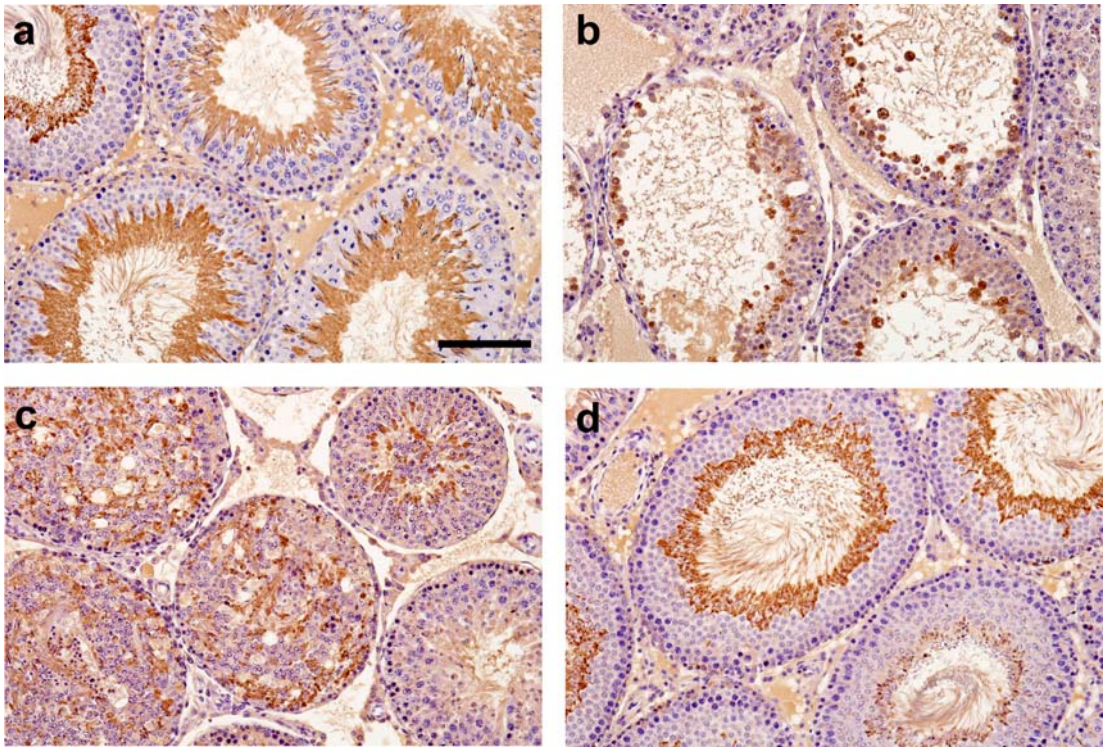


Figure 6-11 Testis sections from a) control testis, and b,c,d) testis treated with glycerol, immunostained with an antibody to seminiferous tubule conditioned medium (brown) and counterstained with haematoxylin (blue). Scale bar represents 100µm.

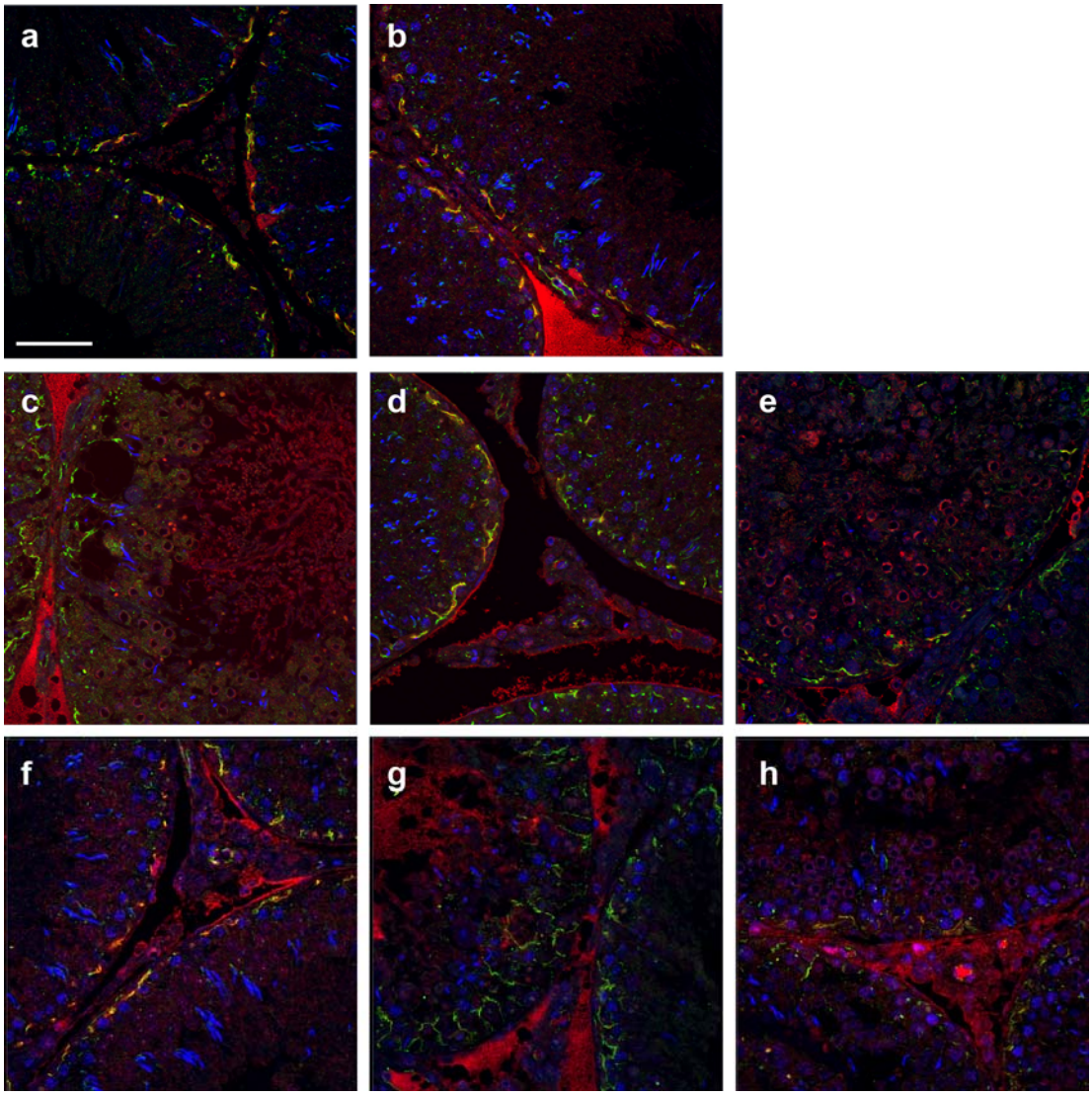


Figure 6-12 Detection of ZO-1 (green) and occludin (red) with Dapi nuclear counterstain (blue), on testis sections from a,b) a control testis, c-h) testes injected with glycerol. Scale bar represents 50µm.

Glycerol treatment was investigated due to its proposed effect on the blood-testis barrier. Co-localisation of the tight junction proteins, occludin and ZO-1 was assessed by immunofluorescence. Figure 6-12 a and b show sections from a control testis used in the glycerol study and illustrate the co-localisation of ZO-1 and occludin at the site of the BTB. Panels c-h show sections taken from testes treated with glycerol. The co-localisation of occludin and ZO-1 can be seen in tubules in panels d and f, which are comparable to the control testes. Panels c, e, g, and h show

localised effects of glycerol treatment which seems to be causing vacuolation (c), and a loss of occludin from the site of the BTB (c, e, g). In panels c, e, and h, it looks like the occludin protein has moved towards the lumen of the tubules and is located around the germ cells. In panel g, the treatment could also be affecting the distribution of ZO-1 as the protein now appears localised in Sertoli cells, perpendicular to the basement membrane rather than paralleling the basement membrane.

The function of the BTB was investigated using the biotin tracer. Testis sections stained to show localisation of the tracer and occludin are shown in Figure 6-13. Control tissue in panel a shows the localisation of the BTB (occludin) and the biotin tracer localised to the interstitum and around the germ cells up to the site of the BTB. Figure 6-13 b and c, again illustrate the localised effect of the glycerol treatment. In panel b, occludin has been lost from the site of the BTB and the biotin tracer can be found throughout the tubules. In panel c, the treated testis section is comparable to the control tissue in panel a, with the tracer localised to the interstitum, and occludin present.

These results suggest that treatment with glycerol does cause damage to the BTB (specifically to occludin), but the effect is localised to the site of injection and is not generalised.

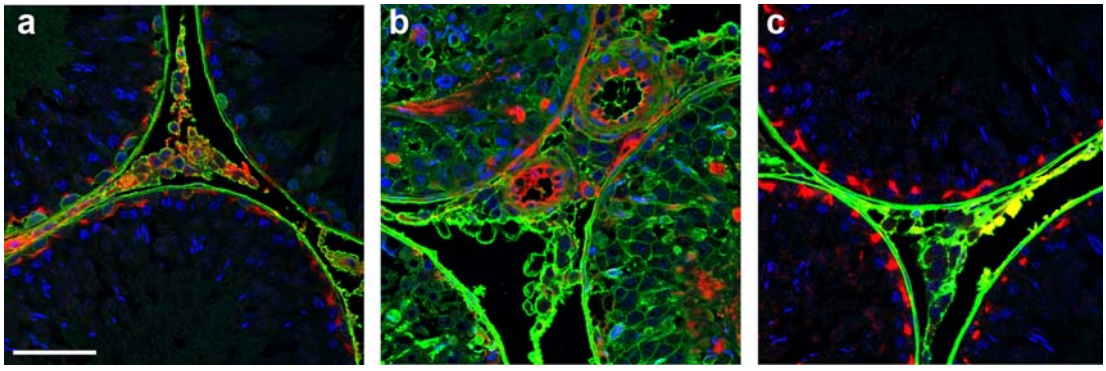


Figure 6-13 Detection of biotin tracer (green) and occludin (red) with Dapi nuclear counterstain (blue), on testis sections from a) a control testis, b,c) testes injected with glycerol. Scale bar represents 50µm.

6.3.2.2 Analysis of Protein Leakage

Leakage of proteins from STs into IF, following glycerol treatment was investigated using Western blotting with the STCM antibody, and Coomassie stained gels. The Western blot is shown in Figure 6-14. From the Western blot, there were no obvious differences in proteins present in the IF samples from control and treated testes. Three bands were quantified, and the graphs for the 45kDa and 39kDa proteins agree with this observation, with no significant differences between control and treated samples. The 15kDa protein band was also quantified, and showed significantly more protein in IF samples collected from testes treated with glycerol. This suggested that the protein could be leaking out of the damaged tubules into the IF.

The Coomassie stained gel with IF samples from control and glycerol treated rats is shown in Figure 6-15. As with the Western blot, there did not appear to be any obvious differences in the protein bands in IF from control and treated testes on the Coomassie gel. Quantification of six proteins, however, showed different results from the Western blot. With proteins of 45kDa, 39kDa, 28kDa, 15kDa, and 12kDa in size, there was a trend for an increase in the samples from glycerol treated testes, but no significant differences. The final band quantified, 27kDa, suggested a slight decrease in concentration following glycerol treatment, although this was, again, not statistically significant. These results suggested a trend for some leakage of proteins from STs following glycerol treatment. The lack of statistical significance of these results could be due to the localised nature of the effect of the treatment. Figure 6-11, Figure 6-12, and Figure 6-13 all show that there are still some unaffected tubules present in the glycerol treated testis, and so protein leakage is likely to only occur from the small number of damaged tubules.

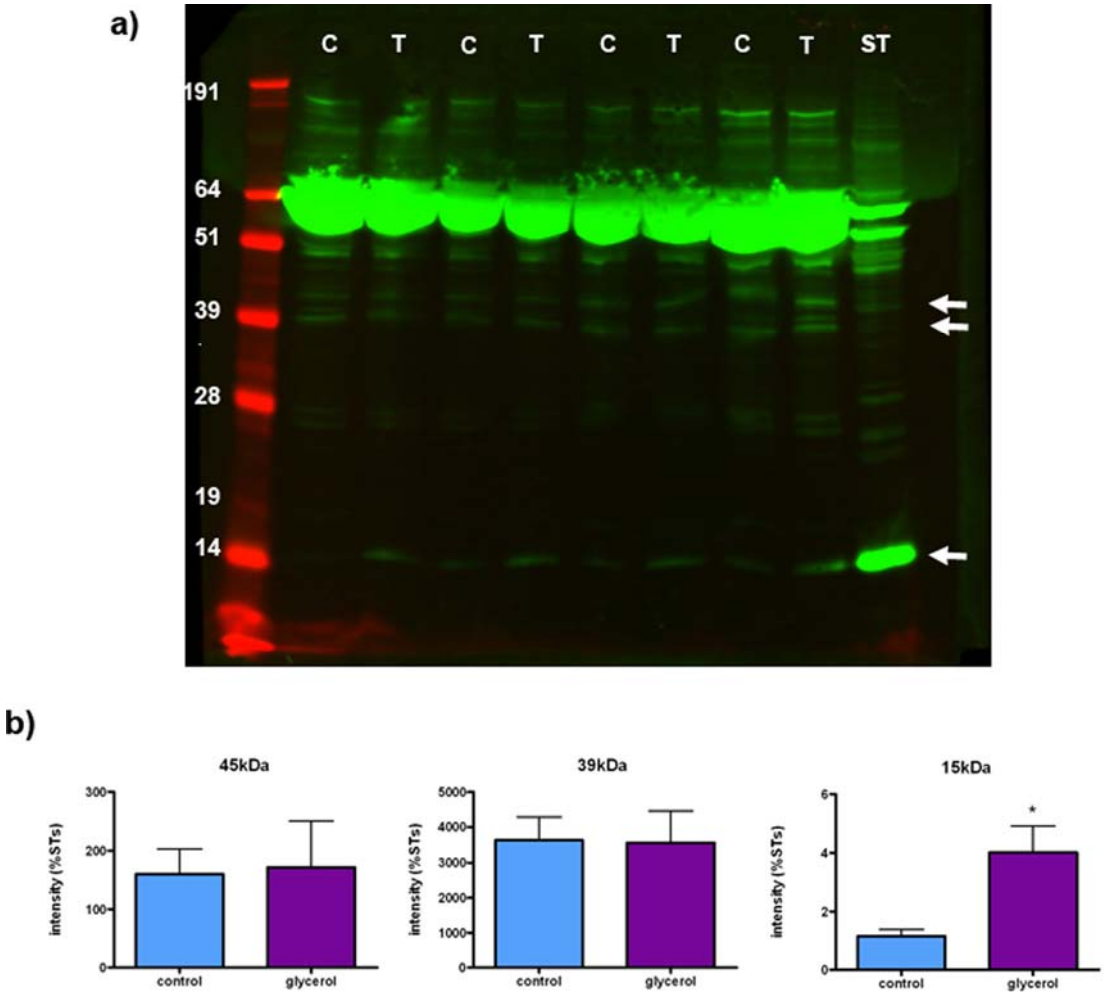


Figure 6-14 a) Western Blot with antibody to STCM, showing interstitial fluid samples from control (C) and glycerol treated (T) testes and control homogenised seminiferous tubules (ST). Arrows indicate examples of protein bands quantified. b) Graphs showing quantification of indicated bands (mean with SEM), with values taken from samples run on one Western blot, expressed as a percentage of STs (n=4 samples/dose group). Statistical analysis, t-test. * $p<0.05$ compared to control samples.

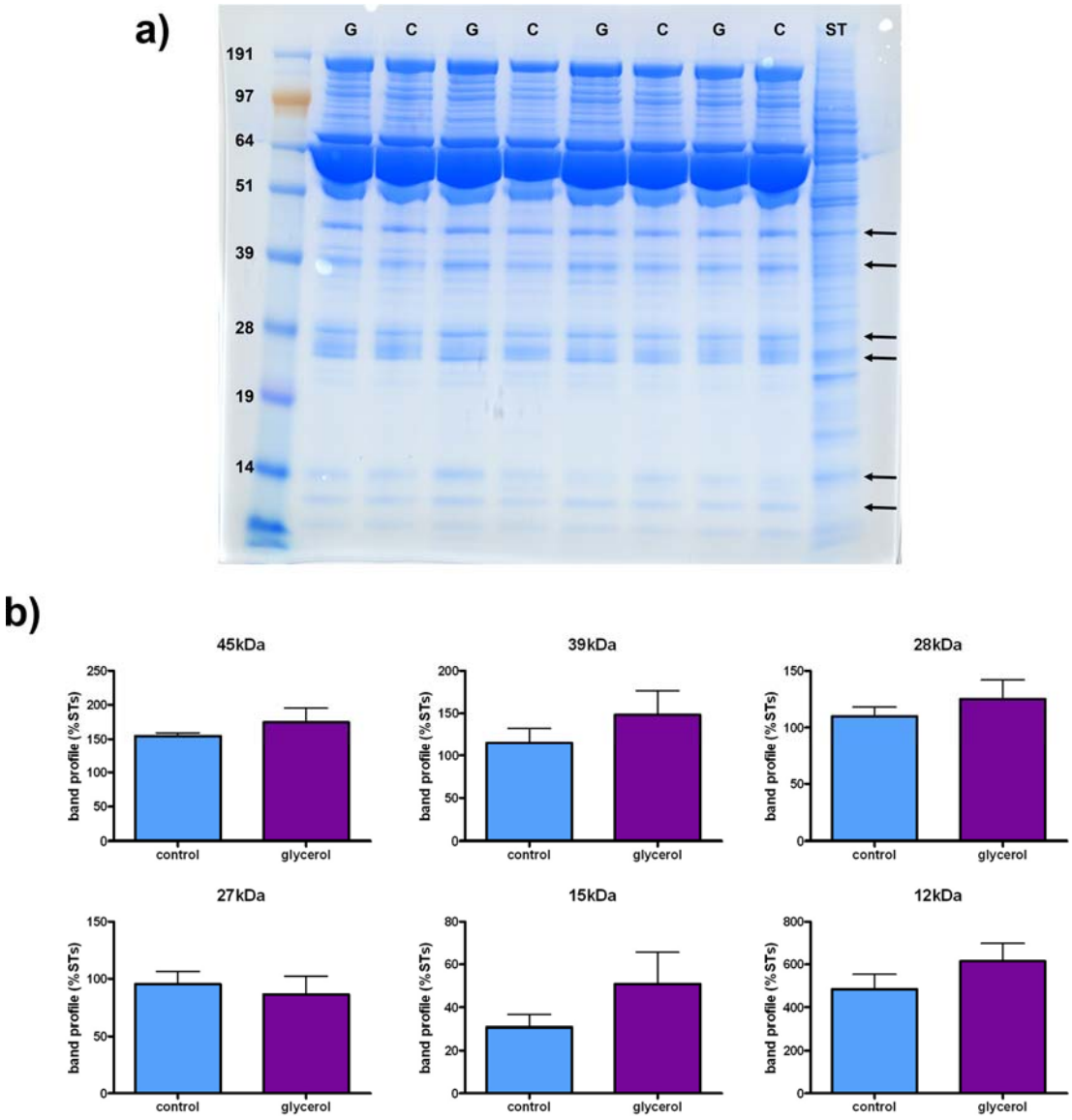


Figure 6-15 a) Coomassie stained gel, showing interstitial fluid samples from control (C) and glycerol treated (T) testes and control homogenised seminiferous tubules (ST). Arrows indicate examples of protein bands quantified. b) Graphs showing quantification of indicated bands (mean with SEM), with values taken from samples run on one gel, expressed as a percentage of STs (n=4 samples/dose group). Statistical analysis, t-test, showed no significant differences.

6.3.3 TGF-β3 Treatment

6.3.3.1 Analysis of Damage

Testis sections from testes treated with TGF-β3, and control testes were immunostained with the general antibody to seminiferous tubule proteins (STCM), to analyse the damage caused by the treatment. Figure 6-16 shows a control testis section (a) and two pictures of a treated testis section (b,c). The results showed that some damage is present in tubules in the treated testis, but most of the tubules appeared normal. This suggested that the TGF-β3 treatment, like the glycerol treatment, has had a localised effect on the seminiferous tubules near the site of injection.

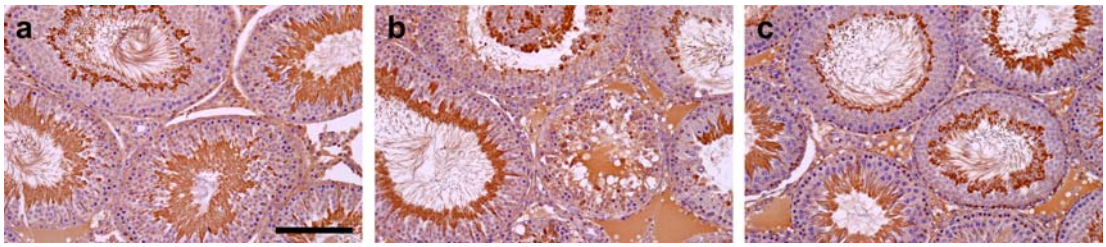


Figure 6-16 Testis sections from a) control testis, and b,c) testis treated with TGF-β3, immunostained with an antibody to seminiferous tubule conditioned medium (brown) and counterstained with haematoxylin (blue). Scale bar represents 100μm.

The state of the blood-testis barrier following treatment with TGF-β3 was investigated by immunofluorescence co-staining of ZO-1 and occludin, and using the biotin tracer. Figure 6-17 shows the co-localisation of occludin and ZO-1 in control tissue (a), and from a TGF-β3 treated testis (b,c). As shown before, ZO-1 and occludin clearly co-localised at the site of the BTB in control tissue (panel a). The tubules in panel c, taken from a TGF-β3 treated testis, are comparable to the control tissue, showing that the integrity of the BTB is still intact in some areas of the testis. Panel b shows another area of a TGF-β3 treated testis, where damage to the tubules was identified (Sertoli cell vacuolation), and where there was apparent loss of occludin protein from the site of the BTB. This supported the results in Figure 6-16 which suggest that TGF-β3 treatment has had a localised effect on the testis.

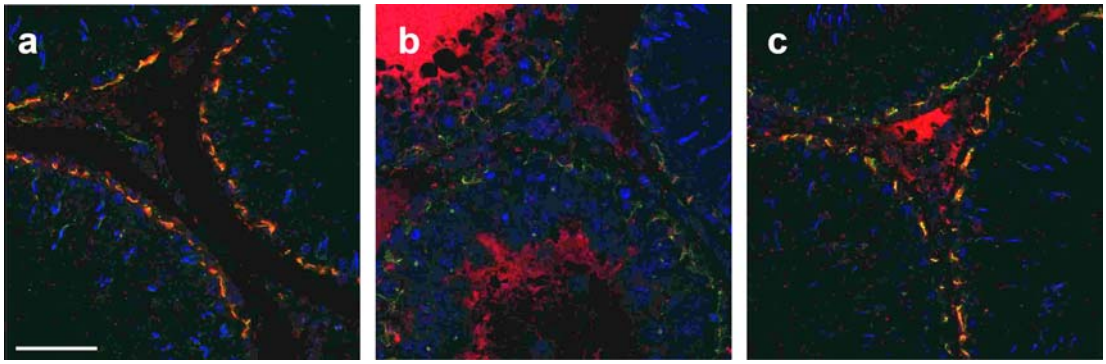


Figure 6-17 Detection of ZO-1 (green) and occludin (red) with Dapi nuclear counterstain (blue), with testis sections from a) a control testis, b,c) testes injected with TGF-β3. Scale bar represents 50μm.

The biotin tracer was used to analyse BTB function. Results are shown in Figure 6-18. As shown before, in control tissue (a), the biotin tracer was restricted to the interstitial compartment and in STs up to the site of the BTB (shown by localisation of occludin) as the BTB is fully functional. Figure 6-18b and c, show STs from a TGF-β3 treated testis. In panel b, the tracer appears to have penetrated the tubules, but occludin still seems to be present. On comparison to some of the control tissue presented in chapter 4, this result could be one of the natural variations seen with the biotin tracer and probably does not represent loss of function of the BTB. In Panel c, two tubules appear to have occludin protein, but the third does not (indicated by *). This could be due to the stage of the tubule (occludin has been reported to be absent at stage VIII (Li et al., 2006)) or an effect of the TGF-β3 treatment. Either way, the biotin tracer has not penetrated this tubule, suggesting that components of the BTB are still present, forming a functional barrier.

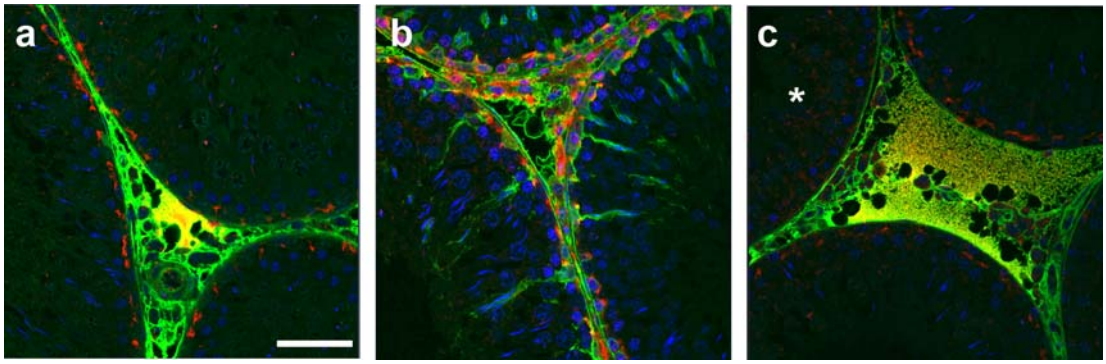


Figure 6-18 Detection of biotin tracer (green) and occludin (red) with Dapi nuclear counterstain (blue), with testis sections from a) a control testis, b,c) testes injected with TGF-β3. Scale bar represents 50μm.

6.3.3.2 Analysis of Protein Leakage

Protein leakage from STs into IF following TGF-β3 treatment was evaluated using Western blotting with the STCM antibody and Coomassie stained gels. Figure 6-19 shows IF samples from control testes and testes treated with TGF-β3 run on a Western blot with the STCM antibody. From the Western blot, there appeared to be some differences in the proteins present in each sample but there were no clear differences between samples from control testes, and TGF-β3 treated testes. Quantification of two protein bands suggested a trend for a decrease in the amount of 45kDa and 15kDa proteins in IF from TGF-β3 treated testes. This result was unexpected, as the TGF-β3 treatment did appear to have some effect on some of the tubules so a slight increase in proteins would have been expected in IF from treated testes rather than a decrease. There could have been problems loading the samples on the gel, as there does seem to be a lot more protein in the first control sample run on the blot compared to all of the other samples, and this could have affected the quantification.

In order to confirm this effect, and look at a wider range of proteins, IF samples from control and treated testes were run on Coomassie stained gels. From the gel, shown in Figure 6-20, there appeared to be no consistent differences between the proteins present in IF from control and treated testes. Quantification of six protein bands

showed differing results. Analysis of the 45kDa and 39kDa bands suggested no differences in the amount of protein in control and treated samples. However, the other four proteins, 28kDa, 25kDa, 15kDa, and 12kDa, all showed a trend for an increase in the protein in IF samples from TGF-β3 treated testes. These results suggest that the treatment could be having some effect on the leakage of proteins from STs into IF, but due to the localised effect of the treatment in a small area of the testis, the results are not significant because proteins are not leaking out of all the tubules.

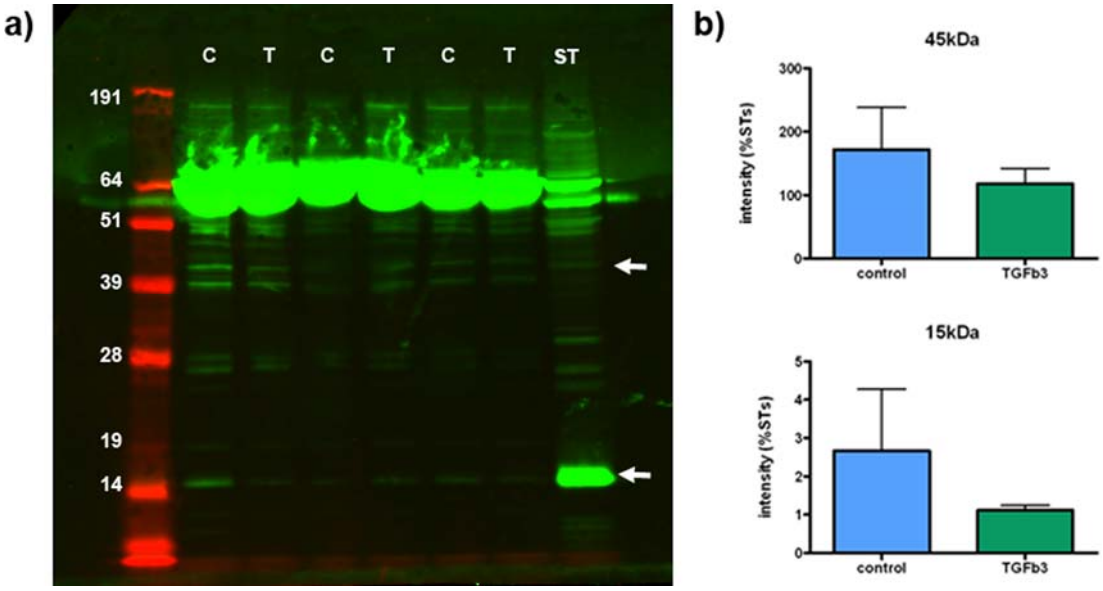


Figure 6-19 a) Western Blot with antibody to STCM, showing interstitial fluid samples from control (C) and TGF-β3 treated (T) testes and control homogenised seminiferous tubules (ST). Arrows indicate examples of protein bands quantified. b) Graphs showing quantification of indicated bands (mean with SEM), with values taken from samples run on one Western blot, expressed as a percentage of STs (n=3 samples/dose group). Statistical analysis, t-test, showed no significant differences.

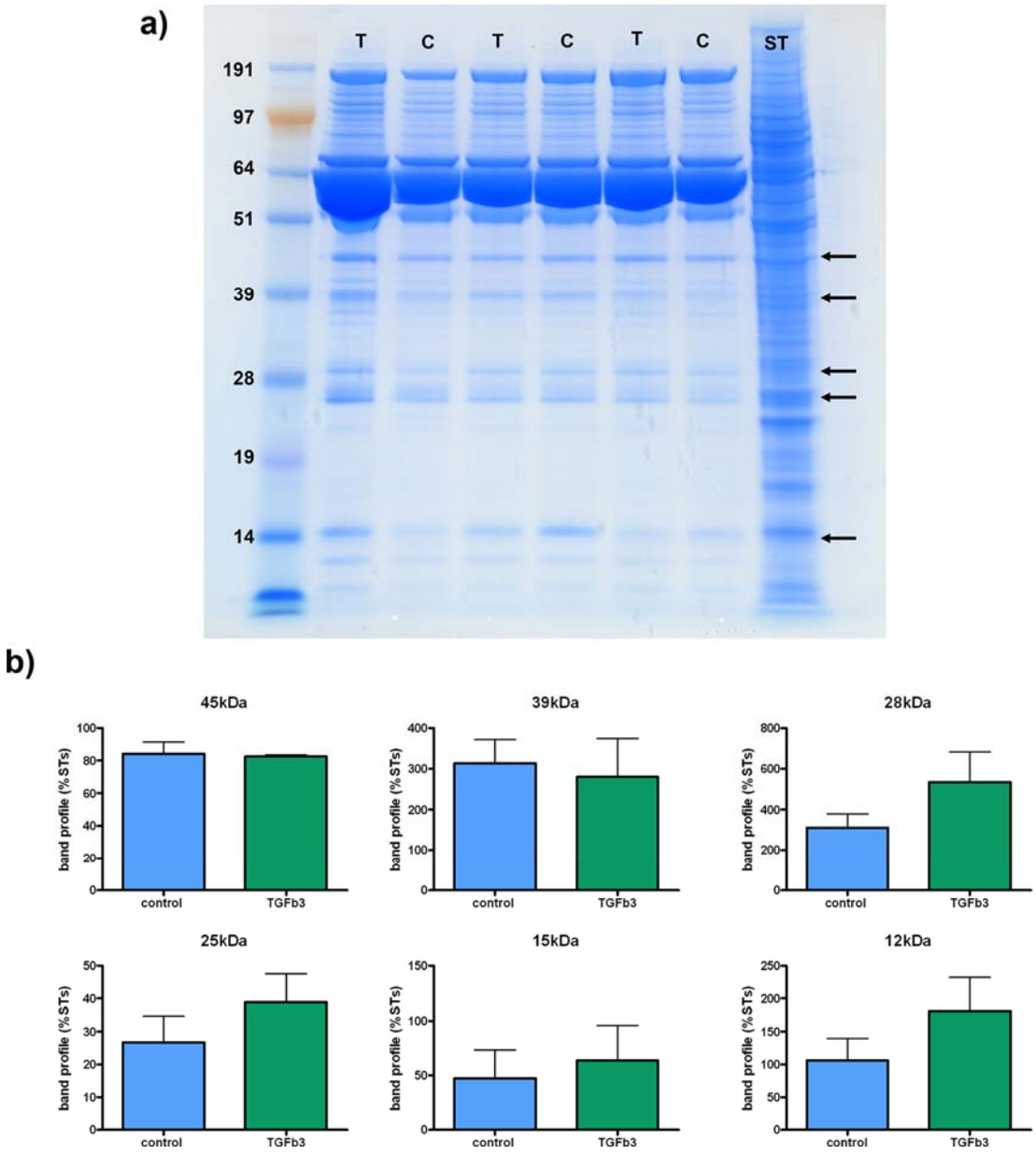


Figure 6-20 a) Coomassie stained gel, showing interstitial fluid samples from control (C) and TGF-β3 treated (T) testes and control homogenised seminiferous tubules (ST). Arrows indicate examples of protein bands quantified. b) Graphs showing quantification of indicated bands (mean with SEM), with values taken from samples run on one gel, expressed as a percentage of STs (n=3 samples/dose group). Statistical analysis, t-test, showed no significant differences.

6.4 Discussion

The aim of this chapter was to further investigate the hypothesis generated at the end of chapter 5, suggesting that proteins leak from seminiferous tubules into interstitial fluid only following loss of integrity of the blood-testis barrier. Three approaches were taken to specifically address this.

The first approach was a physiological approach looking at the development of the BTB and the presence of proteins in interstitial fluid before and after the barrier has formed. Immunofluorescent co-staining for occludin and ZO-1 showed the presence of occludin protein localised to the site of the BTB in the tubules at day 18 whereas it was completely absent from tubules at day 10 and day 15. This agrees with the reported development of the BTB, as the barrier has been shown to form between days 15-19 in the rat (Vitale et al., 1973; Russell et al., 1989). ZO-1 was also present at the BTB from day 18, but some ZO-1 was also present at day 10 and day 15 in a network pattern around the germ cells. This difference in localisation could suggest a role for ZO-1 in germ cell-Sertoli cells interactions. It may also be due to the different ZO-1 isoforms. The ZO-1α^+ isoform was reported to be predominantly expressed during puberty, while ZO-1α^- is predominantly expressed in adulthood (Pelletier et al., 1997). The differing localisation of the protein as the testes develop could be due to a change in the expression of the different ZO-1 isoforms.

Immunofluorescent co-staining for the adherens junction proteins, N-cadherin and β-catenin as well as the biotin tracer technique were also used to assess development of the BTB. N-cadherin appeared to be present at all of the ages investigated, with a network pattern around the germ cells at day 10 and 15, and moving out to the site of the BTB at day 18 and comparable to adult by day 25. β-catenin appeared to be present at day 15 onwards, localised around the germ cells. The localisation of β-catenin at day 25 is comparable to the adult, where it is clearly found localised to the spermatocytes. These results suggest roles for N-cadherin and β-catenin in early testis development, but localisation to the site of the BTB at around day 18,

supporting the results seen with ZO-1 and occludin, and the reported age for barrier formation in the literature.

The biotin tracer technique was used to assess the functional development of the BTB. The results were unexpected as the biotin tracer appeared to be localised to the interstitum at day 10, 15, 18 and 25. Following tracer studies reported in the literature, using peroxidase and lanthanum techniques (Vitale et al., 1973), and the BTB protein localisation results, the biotin tracer was expected to localise to the interstitum only following BTB formation at day 18. The observations could indicate a barrier role for the basement membrane during early testis development.

Expression of the genes for the BTB proteins, occludin, claudin-11, JAM-A, ZO-1, N-cadherin, and β-catenin, was also investigated across the age range. The results were variable, probably due to the small sample numbers, although at these stages in development there could also be large natural variability between different animals. A statistically significant increase in claudin-11 and ZO-1 mRNA expression was observed at day 25, in comparison with adult. mRNA expression of N-cadherin and JAM-A did not appear to change over the age range. mRNA expression of occludin was variable, and β-catenin expression was increased at day 10 in comparison to adult. The mRNA expression data does not fit in with the protein data, and the variability does not allow conclusions to be drawn.

Analysis of proteins in interstitial fluid samples collected from animals at the different ages was performed using Western blotting with the STCM antibody, and Coomassie stained gels. Immunohistochemistry was first carried out with the STCM antibody to evaluate whether it detected proteins present in the testis of younger animals. A range of proteins were detected across the age range, although not all the proteins present in the adult testis appeared to be present in the younger testes.

Western blotting with the STCM antibody to investigate proteins present in IF samples at the different ages showed different proteins present at different stages of

development. Quantification of the protein bands showed variability across the age range for the 45kDa, 39kDa and 28kDa proteins. The 15kDa band illustrated a limitation of this approach, as it was found in higher amounts in IF from adults but little or no protein was present at the earlier ages. This indicated that the 15kDa protein and potentially many more were not expressed in the seminiferous tubules until later in development/adulthood. Isolated seminiferous tubules from the age range were also analysed by Western blotting with the STCM antibody. The results were similar, with many more proteins present in STs from adult rats compared to STs from younger rats. Again this was supported by quantification of the 15kDa band, which seemed to appear in STs from day 25. The same effect was also noted when the IF samples were run on Coomassie stained gels. Quantification of six protein bands showed variability in the amounts of proteins present in IF, and suggested some proteins expressed late in development (day 25) and adulthood are likely to be Sertoli cell derived proteins, such as the 39kDa and 28kDa. The results for the 15kDa band did not show the same result as the Western blots, with the highest concentration of the protein observed in day 18 samples. However this could be due to problems loading the gel, or the small number of samples assessed.

Due to the problems with IF protein analysis due to some proteins being expressed at later stages of development/in later stage germ cells, three specific germ cell proteins were investigated. Calpastatin was shown to be expressed in spermatids, and therefore could not be used to show changes in concentration in IF samples before and after BTB formation. DAZL protein is, however, expressed in the pachytene spermatocytes in adults, and can be detected in early spermatocytes from day 10, as shown with immunohistochemistry. This made DAZL an ideal protein to investigate in IF samples from the different aged rats. Western blotting for DAZL, showed a protein band present in all samples about 25kDa in size. Quantification of this band suggested an increased amount present in adult IF samples but the data was variable. It is unclear whether this band is DAZL protein, because it was expected to be 33kDa in size. The bands observed could be proteins containing some of the epitopes recognised by the antibody rather than actually DAZL protein and detection of

DAZL protein could show a different effect. VASA, also expressed in spermatocytes, was also investigated but was not detected in IF samples across the age range.

The physiological approach, to look at the proteins present in the interstitial fluid before and after formation of the BTB proved inconclusive. Protein analysis was difficult due to many of the proteins of interest not being expressed until the later stages of development.

The second approach taken to investigate loss of integrity of the blood-testis barrier and leakage of proteins from STs into IF was intra-testicular injection of glycerol which has been reported to cause disruption of occludin in the BTB (Wiebe et al., 2000). Analysis of the effects of glycerol treatment on the seminiferous tubules by immunohistochemistry with the STCM antibody suggested that the glycerol treatment had a localised effect on the tubules causing disruption to the organisation of the tubules including germ cell loss, and Sertoli cell vacuolation in some areas of the testis sections analysed. Other areas of the sections appeared comparable to the concurrent control testis. These results suggested that the effect is localised to the site of injection, although it was hard to confirm the site of injection in the paraffin embedded tissue. Analysis of further tissue sections throughout the testis could have been carried out to confirm this. The results agreed with the effects of 10% glycerol treatment reported by Igdoura and Wiebe, 1994, who described suppression of spermatogenesis one week following administration of glycerol, with a gradual loss of all types of germ cells by 8 weeks due to premature disengagement and sloughing of germ cells (in 75% of tubules, while 23% appeared normal, and less than 1% were acellular). This study also suggests that a longer time period may be required for the glycerol treatment to exert its effect throughout the testis, and perhaps the testicular samples should have been collected after a longer time period than 48 hours.

The effect of glycerol treatment on the BTB was investigated by immunofluorescence co-staining for ZO-1 and occludin, and using the biotin tracer

technique. The localisation of the tight junction proteins, ZO-1 and occludin was shown to be variable across different tubules from treated testes. As shown with the immunohistochemistry, some tubules were comparable to control tissue, with ZO-1 and occludin localised to the site of the BTB. In other tubules, ZO-1 appeared to remain localised to the BTB, while there was a loss of occludin from this site. In some of these tubules, occludin appeared to have re-localised away from the BTB and to surround germ cells. An effect on ZO-1 was also suggested in some tubules with apparent localisation perpendicular to the basement membrane rather than paralleling the basement membrane. The results seen with the biotin tracer were also variable, with some tubules from glycerol treated testes comparable to control with the tracer localised to the interstitum and up to the site of the BTB, and penetration of the tracer observed in other tubules from treated testes suggesting a loss of function of the BTB. The results suggested that glycerol is having a localised effect on the integrity of the BTB, and appeared to cause disruption by a loss of occludin protein from the site of the BTB.

Protein leakage from STs into IF following glycerol treatment was evaluated by Western blot and Coomassie stained gel analysis of IF samples. Western blotting with the STCM antibody showed a statistically significant increase in the concentration of the 15kDa protein in IF following glycerol treatment, but no statistically significant differences were observed with the 45kDa and 39kDa protein bands also quantified. These results suggested that leakage of some proteins, such as the 15kDa protein, may be occurring following glycerol treatment. Analysis of IF proteins from vehicle control and glycerol treated testes, by Coomassie dye staining showed a trend for an increase in concentration of five proteins analysed (45kDa, 39kDa, 28kDa, 15kDa, and 12kDa) in IF from glycerol treated testes compared to vehicle control treatment. This suggested that proteins are leaking from STs into IF following glycerol treatment, but due to the localised effect of the treatment on the seminiferous tubules, protein leakage is likely to be occurring from only some of the tubules, which could be why the results of the proteins analysed were not statistically significant. Following observations in the literature (Igdoura and Wiebe, 1994),

analysis of proteins in IF following glycerol treatment after a longer period of time might show a greater amount of protein leaking from the STs. Furthermore, only a small number of samples were analysed in this study (4 animals), and increasing the number of samples could provide more concrete evidence.

The results for the glycerol treatment suggested that glycerol has a localised effect on the testes causing a loss of germ cells, and a loss of occludin protein from the BTB. Analysis of proteins present in IF samples suggested a trend for an increase in the amount of some proteins present in IF from glycerol treated testes, suggesting that proteins are leaking from tubules affected by the glycerol treatment.

The third approach taken to investigate the role of the blood-testis barrier in leakage of proteins from STs into IF was intra-testicular injection of TGF-β3, which had been validated by Xia et al., 2008, as a model for BTB disruption. Analysis of the effects of the TGF-β3 treatment on the testes was carried out by immnuohistochemical staining for the STCM antibody. As with the glycerol treatment, the results showed some tubules appeared comparable to the control tissue, but a small number of tubules (from observation) appeared to be damaged. This suggested that the TGF-β3 was having a localised effect at the sites of injection, although it was hard to identify the injection sites following embedding in paraffin wax. To investigate this further, more tissue sections could have been cut from each of the wax blocks and a number of sections across the testis analysed.

The blood-testis barrier was evaluated in the control and TGF-β3 treated testes by immunofluorescence co-staining for occludin and ZO-1, and using the biotin tracer technique. The results showed some tubules in the TGF-β3 treated testes to be comparable to control, with co-localisation of occludin and ZO-1 at the site of the BTB. In some tubules in the treated testes, there appeared to be some loss of occludin from the site of the BTB. This further supported the idea that TGF-β3 had a localised effect, and suggested that it has affected occludin protein. This agrees with results reported by Xia et al., 2006, who noted a reduction in occludin following TGF-β3

treatment, however, they also reported a reduction in ZO-1 protein as well, and there did not appear to be any loss of ZO-1 in these experiments. With the biotin tracer analysis of BTB function, some tubules in the TGF-β3 treated testes had a fully functional BTB, shown by localisation of the biotin tracer to the interstitum and up to the site of the BTB. Penetration of the biotin tracer into some tubules was observed, although this appeared to be between the Sertoli cells, rather than the complete penetration seen following BTB disruption with cadmium chloride treatment (chapter 5). The result observed is comparable to effects seen in some control tissue (chapter 4), suggesting that it is due to the natural variations seen with the tracer. These results suggested that the BTB was still functional following TGF-β3 treatment. As only occludin has been shown to be absent from TGF-β3 treated testes, it could be that other BTB proteins (including ZO-1) could still be present and maintain the integrity of the BTB.

The leakage of proteins from STs into IF following TGF-β3 treatment was investigated using Western blotting with the STCM antibody and Coomassie stained gels. No obvious differences were observed between the protein bands on the Western blot with the STCM antibody in the IF samples from TGF-β3 treated testes compared to control. Quantification of the 45kDa band and 15kDa band showed a trend for a reduced amount of protein present in IF following TGF-β3 treatment. These results were unexpected. If the TGF-β3 treatment has only had an effect on specific components of the BTB (such as occludin) and BTB integrity is being maintained by other proteins (as suggested by the biotin tracer results), proteins would not be expected to leak from the STs into IF, based on the results discussed in chapter 5. However, the amount of protein in IF following treatment would be expected to be comparable to control, rather than a reduction in protein concentration after treatment. The changes observed could be due to inaccurate loading of samples onto the gel, or inadequate mixing of samples prior to loading. Only a small number of samples were used for this analysis and inclusion of a greater number of samples could provide more reliable results.

Quantification of the bands from the Coomassie stained gel showed variable results. There were no differences observed between the amounts of 45kDa, or 39kDa proteins present in IF from TGF-β3 treated testes compared to control. However, trends for an increase in the concentrations of the other four proteins quantified (28kDa, 25kDa, 15kDa and 12kDa) in IF following TGF-β3 treatment were noted. The results for the 15kDa band are particularly interesting as they disagree with the result seen for the 15kDa band on the Western blot, although the amount of 15kDa protein detected in IF on the Coomassie gel (compared to STs) is much higher than that on the Western blot. This could, however, be due to different 15kDa proteins being detected in the two different systems, or potentially more than one protein of 15kDa in size being detected on the Coomassie gel. The results from the Coomassie gel suggested that there could be some protein leaking from the STs into IF following TGF-β3 treatment, although due to the localised effect of the TGF-β3, the effect on the proteins was not statistically significant.

The aim of this chapter was to further investigate the role of BTB integrity in leakage of proteins from seminiferous tubules into interstitial fluid samples. The results presented suggest that the physiological approach to further investigate the protein leakage hypothesis proved inconclusive due to many of the proteins of interest not being expressed until in later stage germ cells and so were not present while the blood-testis barrier was developing. Specific targeting to occludin in the BTB by glycerol treatment suggested some localised loss of integrity of the BTB and a trend for leakage of proteins from STs into IF following treatment. Targeting the BTB by TGF-β3 treatment caused some localised damage but loss of integrity of the BTB was not observed, although there was a localised loss of occludin protein. Analysis of IF protein content showed variable results with a suggestion of some leakage of proteins from STs into IF following TGF-β3 treatment. The results observed with these treatments support the hypothesis that proteins only leak from STs into IF following a loss of integrity of the blood-testis barrier. Further experiments with these treatments, perhaps leaving the damage to develop for a longer period of time

could be carried out to try and obtain wider disruption of the BTB throughout the testis, and thus more significant protein leakage data.

7 Detection of Testicular Damage using Protein Biomarkers

7.1 Introduction

Throughout this thesis, the hypothesis that germ cell derived proteins may leak from seminiferous tubules into interstitial fluid following toxicant-induced testicular damage has been investigated. If proteins do leak from the STs into IF, following toxicological insult, they may leak from the interstitial fluid into the blood and may be detected in blood samples. This would provide a simple way of determining testicular toxicity following treatment without having to look at testicular tissue. This could allow 'early warning' detection of testicular damage as biomarker levels in the blood could be monitored throughout a toxicology study, allowing detection before the histopathological analysis at the end of the study. Assessing the tissue at the end of the study also only shows the state of the tissue at one point in time. A biomarker would allow a profile of the damage across a period of time to be obtained. Table 7-1 details the criteria for a biomarker for testicular toxicity. This table also highlights a second hypothesis, to be investigated in this chapter, in that smaller molecular weight proteins are thought to be more likely to leak out of STs into IF.

Based on these criteria (Table 7-1), potential protein biomarkers of testicular toxicity were selected from a list of testis specific genes, generated from a bioinformatic screen carried out at GSK. A list of gene expression data was generated by Affymetrix Genechip® analysis of rat testicular tissue (RNA was isolated from homogenised whole testes from control rats), with expression of selected genes validated by TaqMan® analysis (GSK internal report). A literature search was carried out to further characterise the genes of interest identified in the Genechip® analysis and confirm testis specificity (GSK internal report). Three proteins were selected from the resulting list; fatty acid binding protein 9 (FABP9) (also known as TLBP or PERF15), a disintegrin and metalloprotease domain 3 (ADAM3) (also known as cyritestin), and Calpastatin (tCAST), primariliy based on the availability of

commercial antibodies (for ease of protein detection), and the molecular weight (to investigate the hypothesis that smaller molecular weight proteins are thought to be more likely to leak out of STs into IF).

Table 7-1 Criteria for a protein biomarker for testicular toxicity.

Criteria	Reason
Low molecular weight	Suspect low molecular weight proteins are more likely to leak out of STs
Abundant	Enough protein must be present to reach the blood system
Testis specific	Protein cannot be present in other tissues or testicular damage will not be detected
Commercially available antibodies to protein	For ease of protein detection and consistency of results
Not usually found in IF/plasma/serum	Protein cannot naturally leak out of tubules
Stable	Once protein leaks from STs, it must be stable enough to leak into the IF/blood for detection

FABP9 is a small transport protein (15kDa) present in the rat perforatorium at the inner acrosomal and outer face of the nuclear envelope of the sperm head, and can be identified in pachytene spermatocytes and spermatids (Oko and Morales, 1994). It is thought to mediate structural rearrangement and stability of the acrosome (Oko and Morales, 1994), as well as having a role in germ cell apoptosis in the developing testis (Kido and Namiki, 2000). ADAM3 is germ cell specific, found in round and elongate spermatids (Linder et al., 1995). It is a transmembrane protein in the acrosomal membrane, and is processed to its active form during sperm transport in the epididymis (Linder et al., 1995). Calpastatin is an inhibitor of calpain, a calcium dependent cysteine protease. The testis specific form (tCAST) has a different N-terminal domain to somatic calpastatin, which causes it to localise to the cytosol (Li et al., 2000). Testis calpastatin is a membrane associated protein present in spermatids, and is thought to have a role in the acrosome reaction and gamete fusion

(Li and Goldberg, 2000). ADAM3 (110kDa precursor) and calpastatin (76kDa predicted) are larger than FABP9 (15kDa) and so could be used to show that only small proteins leak from the seminiferous tubules following toxicant induced damage to spermatogenesis.

Two well-studied germ cell proteins were also investigated as potential biomarkers for testicular toxicity. The selected proteins were VASA and DAZL. VASA is a member of the DEAD-box family of genes, which encodes an ATP-dependent RNA helicase and is expressed specifically in germ cells and involved in regulation of cell proliferation (Tanaka et al., 2000). DAZL (deleted in azoospermia like) is an RNA binding protein expressed in multiple cellular compartments (nucleus and cytoplasm) during development of male germ cells (Reijo et al., 2000). Again both proteins are different sizes, VASA is a larger protein at 76kDa, and DAZL is a smaller protein, 33kDa in size.

All five proteins were investigated by looking at the localisation of the proteins using immunohistochemistry. The ability to detect the proteins in IF samples, and to identify if they leaked out of STs after toxicant-induced damage was analysed using Western blotting.

7.2 Methods

7.2.1 Samples

Samples collected throughout the studies detailed previously in this thesis were used for evaluating potential protein biomarkers. Fixed testes, interstitial fluid, and snap-frozen testis samples from the cadmium chloride, MAA, and DNB studies were used for the main investigations. Fixed tissue, and interstitial fluid samples from the glycerol and TGF-β3 studies, and fixed tissue, snap-frozen tissue, and isolated seminiferous tubules from the development study were used for additional investigations.

Detailed descriptions of treatments and sample collection methods are presented in chapter 3. Information regarding the design and purpose of the main studies is included in chapter 5 (cadmium chloride, MAA, DNB) and chapter 6 (development, glycerol, TGF-β3).

7.2.2 Immunohistochemistry

Immunohistochemistry with antibodies to the proteins of interest (DAZL, VASA, ADAM3, Calpastatin, FABP9), was carried out to investigate the localisation of the proteins in the testis. The DAB protocol described in section 3.5 was used.

7.2.3 Western Blotting

Western blotting was used to investigate whether the proteins of interest are present in interstitial fluid and ST samples. Antibodies to DAZL, VASA, ADAM3, Calpastatin and FABP9 were used, following the methods described in sections 3.8 and 3.10. Where necessary, blocking peptides were purchased and run alongside samples on Western blots to try and confirm the identity of the protein bands observed.

7.2.4 TaqMan® Q-PCR

Snap-frozen tissue collected from the cadmium chloride, MAA, DNB, and development studies was used for RNA isolation. cDNA was made from the isolated RNA samples, and used in the TaqMan® reaction with primers and probes for the FABP9 gene. Detailed methods are described in section 3.11.

7.3 Results

7.3.1 DAZL

Immunohistochemistry to show DAZL was used in chapter 5, as a good marker of toxicant induced damage. Figure 7-1 shows testis sections taken from animals treated with one of the three doses of the main testicular toxicants used in this thesis (cadmium chloride, MAA, DNB). DAZL was clearly present in the pachytene spermatocytes and residual bodies towards the lumen, as seen in the control tissue. With low dose toxicant treatment, a reduction in DAZL staining was suggested following MAA and DNB treatment, which caused a loss of pachytene spermatocytes. With low dose $CdCl_2$ treatment (1mg/kg) no changes were identified, and there did not appear to be any damage to the tubules. Following high dose toxicant treatment, there was a reduction in DAZL staining with all three toxicants. This reduction in DAZL staining, from observation, looked to be more marked following high dose treatment compared to low dose treatment with MAA and DNB. This property and the germ cell specificity of DAZL, along with its small size make it a good potential biomarker candidate.

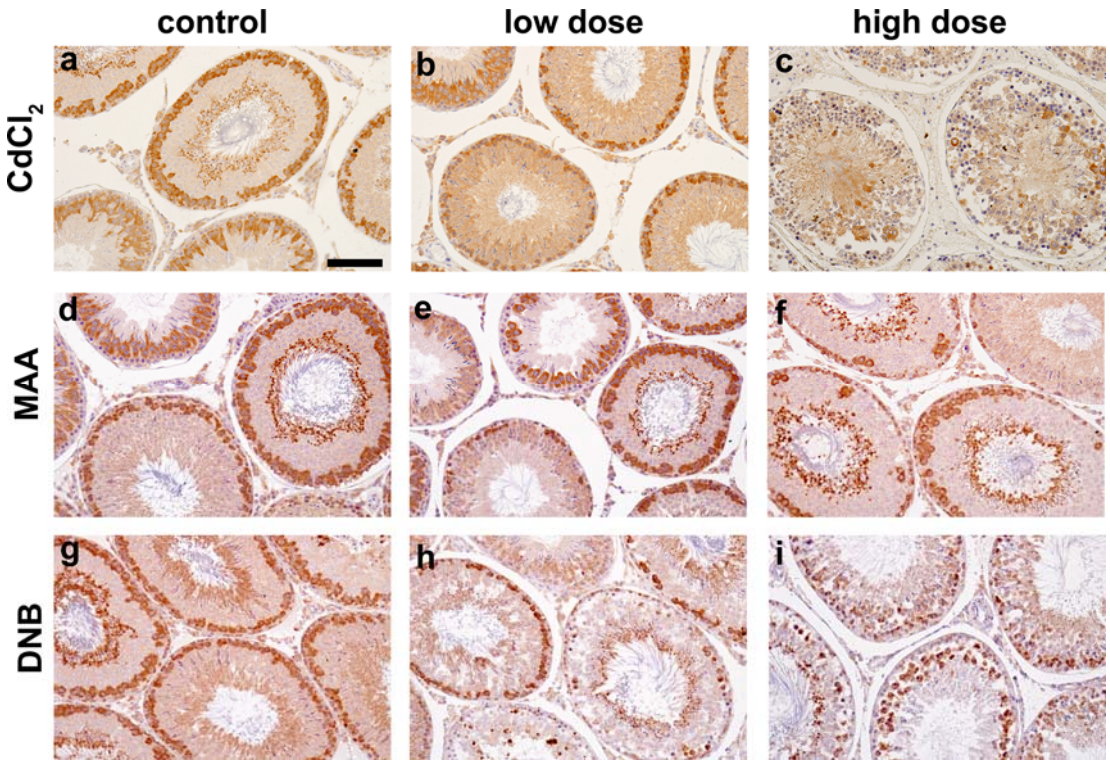


Figure 7-1 Testis sections from control rats from each toxicant study (a, d, g) and rats treated 24 hours earlier with a low dose of toxicant (1mg/kg $CdCl_2$ (b), 200mg/kg MAA (e) or 25mg/kg DNB (h)) or a high dose of toxicant (3mg/kg $CdCl_2$ (c), 650mg/kg MAA (f) or 50mg/kg DNB (i)), immunostained to show DAZL (brown) and counterstained with haematoxylin (blue). Scale bar represents 100µm.

For DAZL to be used as a biomarker for testicular toxicity, it must leak out of STs into IF, following toxicological insult to the testes. IF samples from the three toxicant studies were run on Western blots and probed with the DAZL antibody. Figure 7-2 shows IF samples from the cadmium chloride study. Three different sized bands can be identified on the Western blot. The first, seen in all samples at a size of 64kDa is albumin, likely to be detected due to the large amount of this protein in IF samples, and its 'sticky' nature. A band around 39kDa in size can be seen in the ST sample, and bands about 25kDa in size can be identified in the IF samples. These bands are thought to be DAZL, because they are around the expected size of DAZL (33kDa) and the detection system is picking up these bands specifically. The size discrepancy between the bands in the IF samples and the ST sample could be due to the protein being processed and shortened when leaking out of the germ cells/seminiferous

tubules, resulting in a protein about 25kDa in length. Further explanations could be due to species differences in the protein size or the gel system used to separate the proteins. Alternatively the protein bands observed on the Western blot could be proteins containing some of the epitopes recognised by the antibody which are naturally present in interstitial fluid, and not DAZL protein. The minimal amount of protein present in any of the samples, perhaps, supports this explanation. These bands were quantified and the results shown in Figure 7-2b. The graph shows no significant differences in the amount of DAZL protein present in IF samples from animals treated with the four different $CdCl_2$ doses. This, and the minimal amount of protein detected, suggests that DAZL is not leaking out of STs into IF, following cadmium chloride induced damage, as a larger amount of protein would be present in the IF samples from the 3mg/kg dose group if this were the case (protein leakage suggested at this dose in chapter 5).

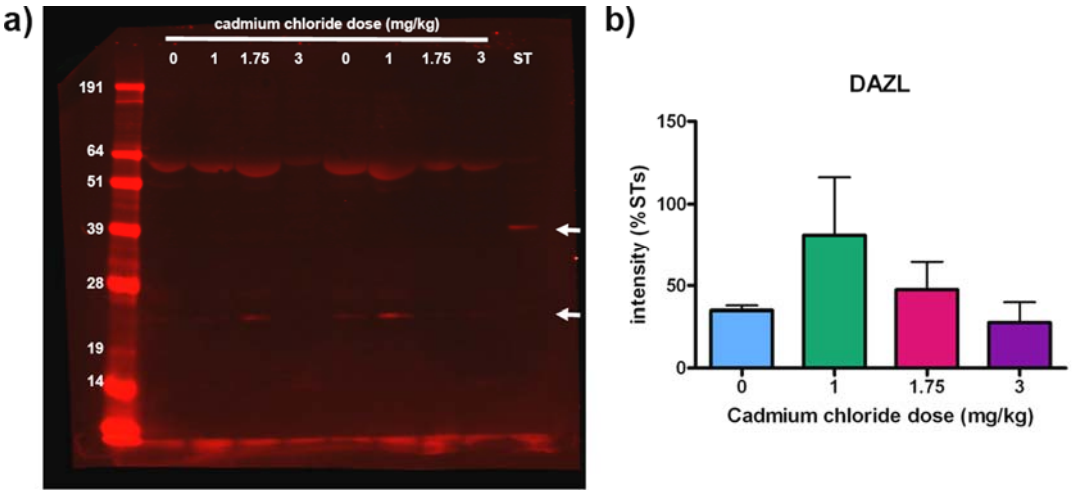


Figure 7-2 Representative Western Blot probed with DAZL antibody, showing interstitial fluid samples from control rats and animals treated with 1, 1.75 or 3.0mg/kg $CdCl_2$ and control homogenised seminiferous tubules (ST). Arrows indicate DAZL protein bands. (b) Graphs showing quantification of 25kDa band (mean with SEM), thought to be DAZL with values taken from samples run on two separate Western blots (n=4 samples/dose group), expressed as a percentage of STs (39kDa band used for normalisation). Statistical analysis, one-way ANOVA followed by Tukey post-test showed no significant differences.

IF samples from the MAA and DNB studies were also run on Western blots and probed with DAZL antibody. The results are shown in Figure 7-3. Again, bands at about 25kDa were detected in IF samples, and a band of about 39kDa in STs were detected. Quantification of the bands on both Western blots, suggest no significant differences in the amount of DAZL protein in IF samples across the MAA dose range, nor across the DNB dose range. This result further suggests that DAZL protein is not leaking out of STs into IF following toxicant induced damage to the germ cells, so DAZL could not be used as a biomarker. The lack of protein leakage with MAA and DNB treatment is not unexpected, following on from the results presented in chapter 5, which suggested that loss of integrity of the BTB is necessary for leakage of proteins, and these treatments specifically target spermatocytes and Sertoli cells, respectively. However, the samples were available, and it is important to further explore the protein profiles of these IF samples, to provide further evidence that loss of integrity of the BTB is needed for proteins to leak from STs into IF.

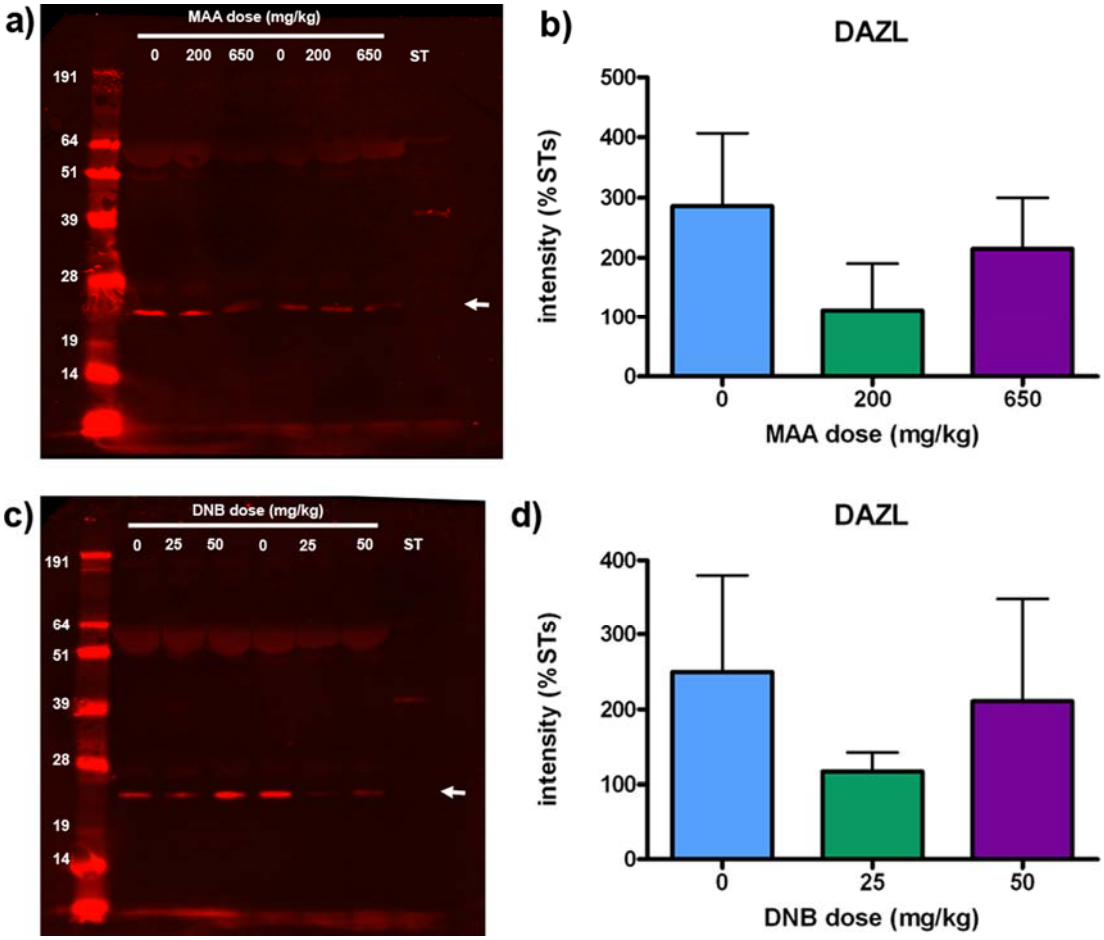


Figure 7-3 Representative Western Blots probed with DAZL antibody, showing (a) interstitial fluid samples from control rats and animals treated with 200 or 650mg/kg MAA and control homogenised seminiferous tubules (ST), and (c) interstitial fluid samples from control rats and animals treated with 25 or 50mg/kg DNB and control homogenised seminiferous tubules (ST). Arrows indicate DAZL protein bands. Graphs showing quantification of 25kDa band (mean with SEM), thought to be DAZL with values taken from samples run on two separate Western blots (n=4 samples/dose group), expressed as a percentage of STs (39kDa band used for normalisation), for (b) MAA samples, and (d) DNB samples. Statistical analysis, one-way ANOVA followed by Tukey post-test showed no significant differences

7.3.2 VASA

The second widely used germ cell protein investigated was VASA. Immunohistochemistry with VASA to show its localisation in tissue from control, low dose and high dose groups from each of the main toxicant studies is shown in Figure 7-4. The control tissue shows that VASA is expressed in more germ cells than DAZL, namely in all spermatocytes and spermatids. Again there was no

difference between control tissue and that from animals treated with low dose cadmium chloride (1mg/kg). There was, however, a loss of some VASA protein with low doses of MAA and DNB, due to their depletion of spermatocytes. With high doses of all three treatments, there was a more marked loss of VASA protein compared to the low dose. Again, this makes VASA a potential candidate biomarker, although it is a larger protein, at 76kDa in size, and small molecular weight proteins are thought to be more likely to leak out of STs into IF.

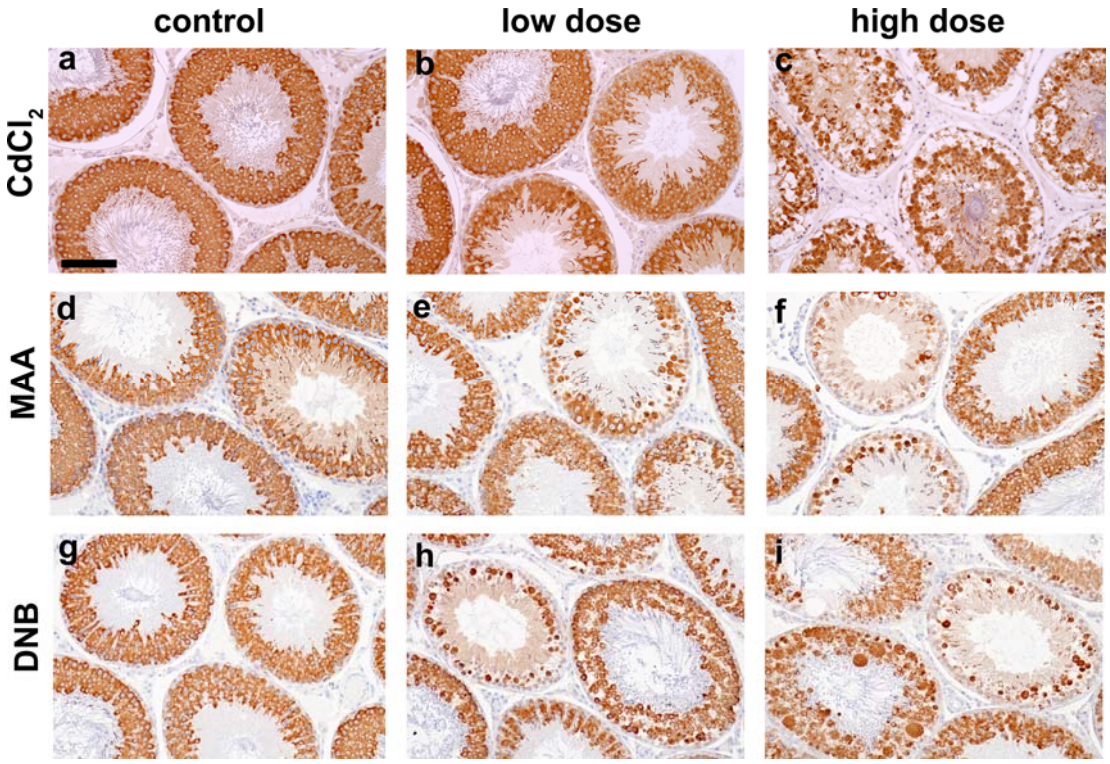


Figure 7-4 Testis sections from control rats from each toxicant study (a, d, g) and rats treated 24 hours earlier with a low dose of toxicant (1mg/kg $CdCl_2$ (b), 200mg/kg MAA (e) or 25mg/kg DNB (h)) or a high dose of toxicant (3mg/kg $CdCl_2$ (c), 650mg/kg MAA (f) or 50mg/kg DNB (i)), immunostained to show VASA (brown) and counterstained with haematoxylin (blue). Scale bar represents 100µm.

To investigate whether leakage of VASA does occur following cadmium chloride treatment, IF samples collected during the studies were run on a Western blot and probed with VASA antibody. A representative Western blot is shown in Figure 7-5. A band of about 76kDa in size was identified in the ST sample and in some IF

samples. A band with a slightly smaller molecular weight was also identified in some lanes, thought to be albumin due to its size of 64kDa. From the blot, VASA appeared to be present in IF samples collected from animals treated with high dose (3mg/kg) cadmium chloride, and in control STs. Quantification of the 76kDa bands from the four separate Western blots required to run all the IF samples collected during the studies is shown in Figure 7-5b. There was significantly more VASA protein identified in IF from animals treated with 3mg/kg $CdCl_2$, suggesting that VASA was leaking out of the STs into IF following high dose cadmium chloride treatment. From the graph, there is also a hint that there was more VASA protein present in IF samples from animals treated with 1.75mg/kg $CdCl_2$, compared to control and low dose, suggesting that there may be a small amount of VASA leaking out with the damage caused by the mid-dose treatment. This dose response is ideal for a biomarker, as it suggests the amount of protein leaking out is proportional to the amount of damage present, so the extent of the damage could be predicted based on the amount of biomarker detected.

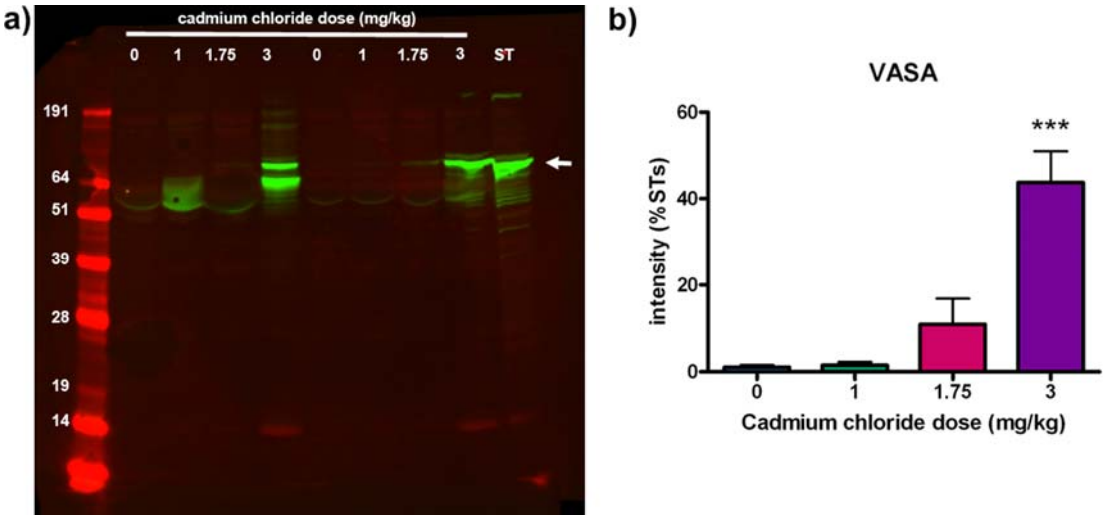


Figure 7-5 a) Representative Western Blot probed with VASA antibody, showing interstitial fluid samples from control rats and animals treated with 1, 1.75 or 3.0mg/kg $CdCl_2$ and control homogenised seminiferous tubules (ST). Arrow indicates VASA protein. b) Graph showing quantification of 76kDa band (mean with SEM), thought to be VASA with values taken from samples run on four separate Western blots (n=7 samples for control, n=5 for 1mg/kg group, n=8 for 1.75 and 3mg/kg groups), expressed as a percentage of the ST sample. Statistical analysis, one-way ANOVA followed by Tukey post-test. *** $p<0.001$ compared to control samples (0mg/kg).

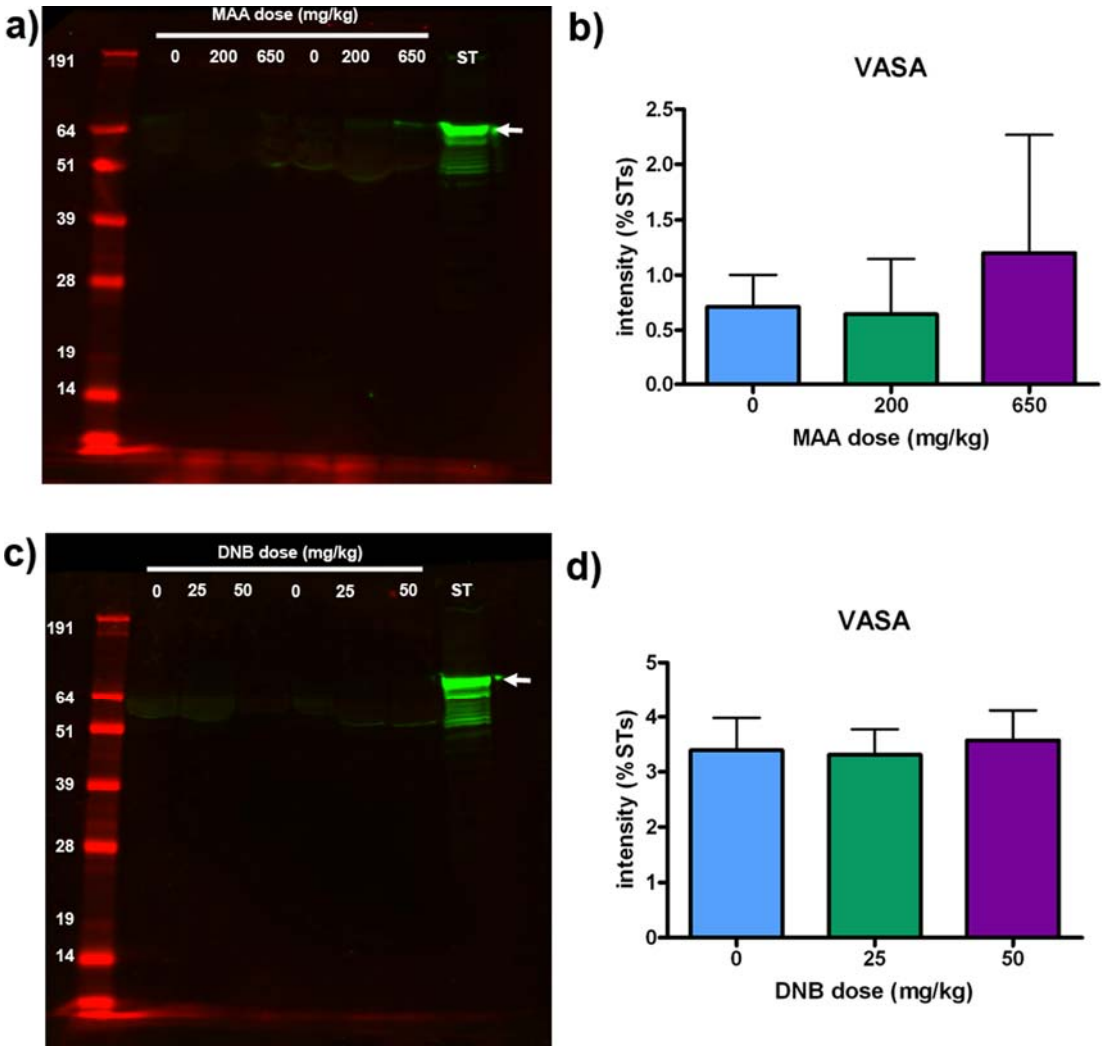


Figure 7-6 Representative Western blots probed with VASA antibody, showing (a) interstitial fluid samples from control rats and animals treated with 200 or 650mg/kg MAA and control homogenised seminiferous tubules (ST), and (c) interstitial fluid samples from control rats and animals treated with 25 or 50mg/kg DNB and control homogenised seminiferous tubules (ST). Arrows indicate VASA protein bands. Graphs showing quantification of 76kDa band (mean with SEM), thought to be VASA with values taken from samples run on two separate Western blots (n=4 samples/dose group), expressed as a percentage of STs, for (b) MAA samples, and (d) DNB samples. Statistical analysis, one-way ANOVA followed by Tukey post-test showed no significant differences

Interstitial fluid samples collected from the MAA and DNB studies were also run on Western blots and probed with VASA antibody, to further investigate the theory that proteins only leak out of STs into IF, following loss of integrity of the BTB. The Western blots are shown in Figure 7-6. As before, a band about 76kDa in size was

seen in ST samples, suggesting VASA had been successfully detected on the blots. The protein did not appear to be present in IF samples from animals treated with vehicle control, or with any dose of MAA or DNB. This conclusion was supported by the quantification, where no significant differences in the amounts of VASA protein can be identified between control and treated samples, and the values detected are likely to be background noise especially when compared with the high levels of VASA protein (40-fold higher) detected for high dose cadmium chloride (Figure 7-5).

7.3.3 ADAM3

The first of the potential biomarker candidate proteins selected from the bioinformatic screen was ADAM3. The localisation of ADAM3 was investigated using immunohistochemistry on testis sections from control animals and from animals treated with 3mg/kg cadmium chloride (Figure 7-7). ADAM3 was expressed in round spermatids in the control tissue. Less ADAM3 protein was evident following high dose cadmium chloride treatment, due to the extensive damage to the organisation of the tubules following treatment.

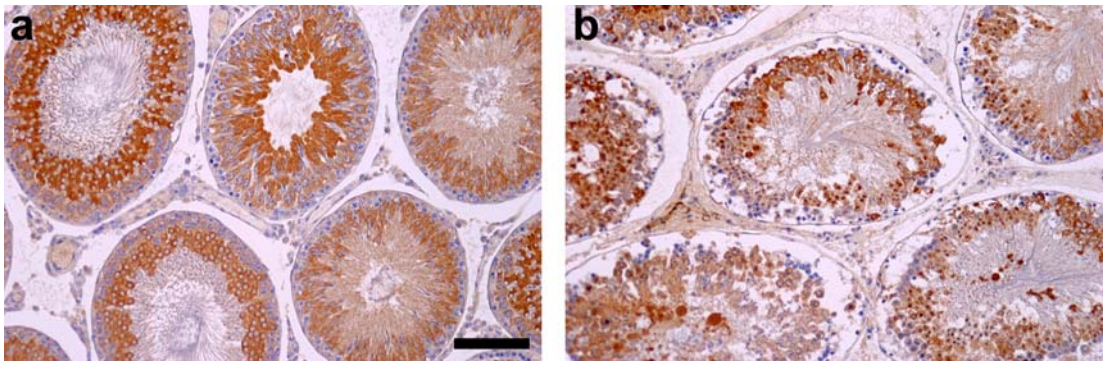


Figure 7-7 Testis sections from a) control (0.9% saline) rat and b) rat treated 24 hours earlier with 3.0mg/kg $CdCl_2$, immunostained with an antibody to ADAM3 (brown) and counterstained with haematoxylin (blue). Scale bar represents 100µm.

The presence of ADAM3 protein in IF samples from control and high dose cadmium chloride treated animals and seminiferous tubules was investigated using Western blotting. Figure 7-8 shows a Western blot probed with ADAM3 antibody. One

protein band was identified in all IF samples, about 64kDa in size. This size corresponds to the size of albumin, a high concentration of which is present in IF samples, and does seem to trap antibodies/proteins on Western blots. Two further bands (about 42 and 28kDa) were also identified in the ST sample. ADAM3, however, is expected to be 110kDa in size (precursor protein), so these protein bands do not appear to be ADAM3, even though the ADAM3 antibody had been used to probe the membrane. Due to a relatively high concentration of antibody being necessary for this Western blot (and the immunohistochemistry) and the unknown nature of the bands detected on the Western blot, ADAM3 was not investigated further as a potential biomarker candidate protein.

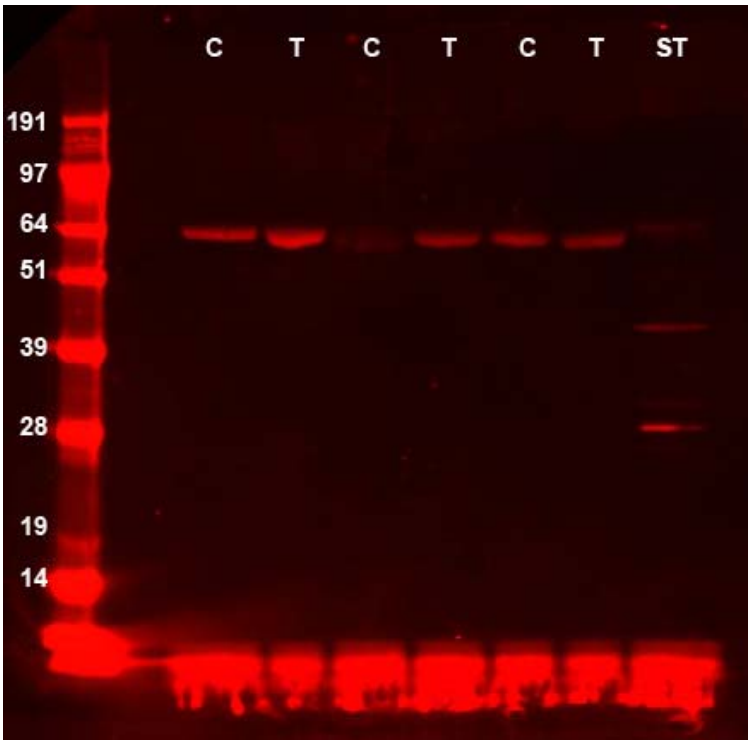


Figure 7-8 Western Blot probed with ADAM3 antibody, showing interstitial fluid samples from control rats (C) and animals treated with 3.0mg/kg $CdCl_2$ (T) and control homogenised seminiferous tubules (ST).

7.3.4 Calpastatin

The second potential biomarker protein to be investigated was calpastatin. The location of the protein was evaluated using immunohistochemistry (Figure 7-9). Calpastatin was expressed in spermatids as seen in the control tissue. Treatment with low doses of all three toxicants did not appear to have any effect on the localisation of calpastatin or the expression of the protein. Although low doses of MAA and DNB have been shown to have an effect on the tubules, both causing a loss of pachytene spermatocytes, as tissue was collected only 24 hours after treatment, these treatments had no effect on the spermatids. Following high dose treatment of all three toxicants, less calpastatin staining was evident, presumably due to the more marked adverse effect the high dose treatments had on spermatogenesis.

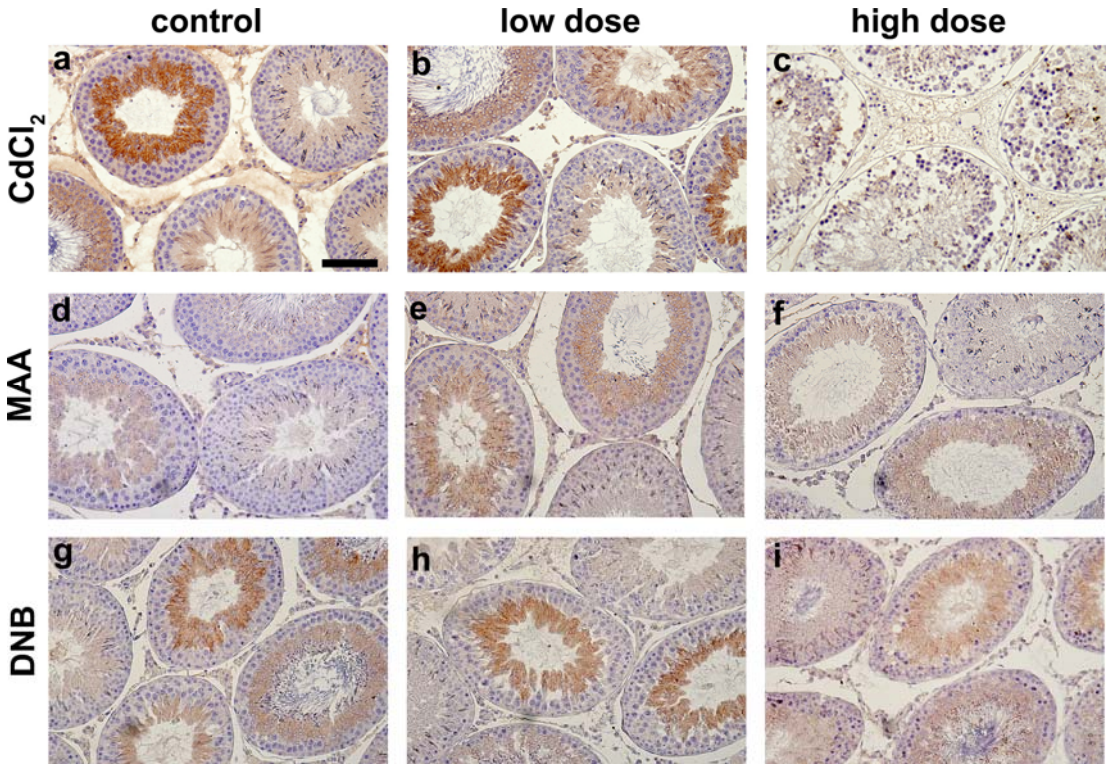


Figure 7-9 Testis sections from control rats from each toxicant study (a, d, g) and from rats treated 24 hours earlier with a low dose of toxicant (1mg/kg $CdCl_2$ (b), 200mg/kg MAA (e) or 25mg/kg DNB (h)) or a high dose of toxicant (3mg/kg $CdCl_2$ (c), 650mg/kg MAA (f) or 50mg/kg DNB (i)), immunostained to show calpastatin (brown) and counterstained with haematoxylin (blue). Scale bar represents 100µm.

A Western blot trial was carried out with IF from control and 3mg/kg $CdCl_2$ treated animals to determine the required concentration of calpastatin antibody (Figure 7-10). Calpastatin protein should be 76kDa in size, although the antibody used should detect bands at 70kDa and 52kDa. The full length protein can also be detected at 110kDa, according to the manufacturers' information. On the Western blot, a prominent band at around 170kDa was seen in both IF samples. Further fainter bands were evident at about 150kDa, 64kDa (albumin), and 39kDa and 14kDa in the sample from the treated animal. In the ST sample, bands at about 150kDa, 39kDa and 25kDa were detected. As none of these protein bands correspond to the expected size of calpastatin, and the lack of availability of a blocking peptide to investigate whether the high molecular weight band seen is calpastatin, the protein was not investigated further as a potential biomarker candidate.

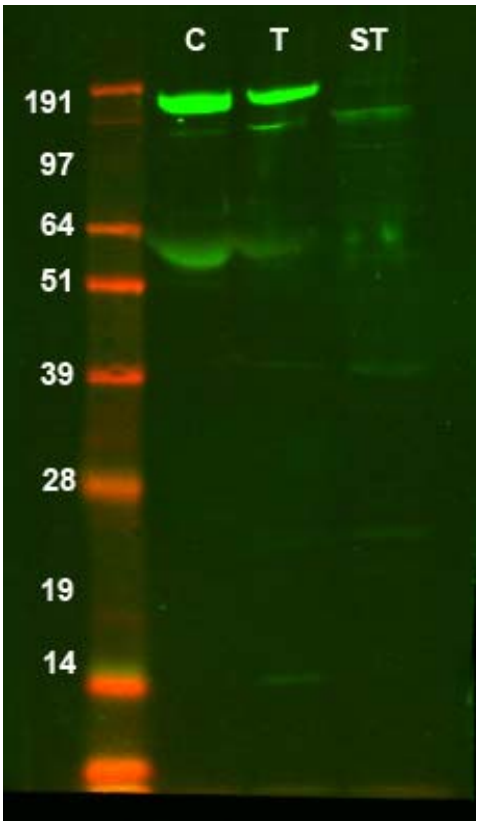


Figure 7-10 Western blot probed with calpastatin antibody, showing interstitial fluid samples from a control rat (C) and an animal treated with 3.0mg/kg $CdCl_2$ (T) and control homogenised seminiferous tubules (ST).

7.3.5 Fatty Acid Binding Protein 9

7.3.5.1 FABP9 Protein Analysis

The final biomarker candidate protein was FABP9. Its localisation is shown in Figure 7-11, although the staining is not very distinct. It appeared to be expressed in spermatocytes and spermatids. Following low dose toxicant treatment, the expression and localisation of the protein did not appear to change. After treatment with high doses of toxicants, the overall level of staining still seemed comparable to the control tissue, even though damage to the tubules and loss of spermatocytes were evident.

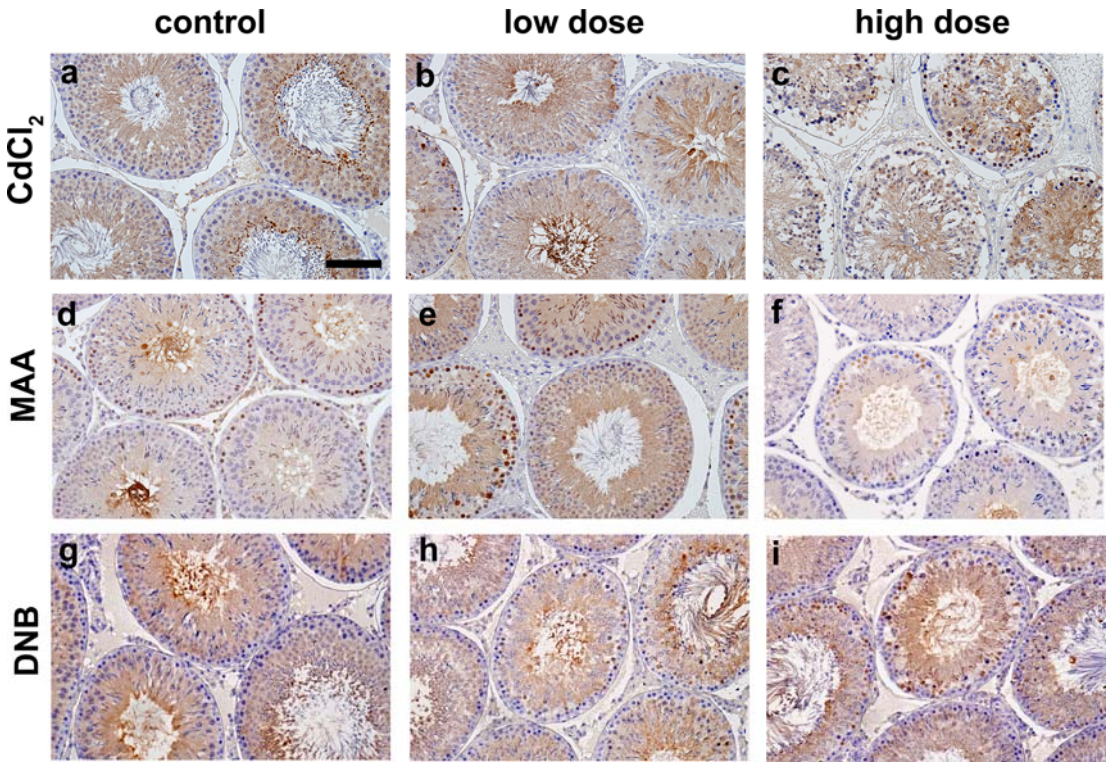


Figure 7-11 Testis sections from control rats from each toxicant study (a, d, g) and from rats treated 24 hours earlier with a low dose of toxicant (1mg/kg $CdCl_2$ (b), 200mg/kg MAA (e) or 25mg/kg DNB (h)) or a high dose of toxicant (3mg/kg $CdCl_2$ (c), 650mg/kg MAA (f) or 50mg/kg DNB (i)), immunostained to show FABP9 (brown) and counterstained with haematoxylin (blue). Scale bar represents 100µm.

The presence of FABP9 in interstitial fluid samples was investigated by Western blotting with an antibody to FABP9. Figure 7-12 shows IF samples collected from animals treated with the various doses of cadmium chloride. A protein band of about

15kDa in size was detected in some IF samples and in the ST sample. This corresponds with the expected size of FABP9 and so suggests that FABP9 protein is being detected in this Western blot. A large band was present in the ST sample, showing that FABP9 protein is present in STs, as expected following the immunohistochemistry results. Bands were also detected in IF samples collected from animals treated with 1.75mg/kg and 3mg/kg $CdCl_2$, and a faint band was evident in IF samples from animals treated with 1mg/kg $CdCl_2$. No bands were seen in IF samples from control animals. This suggests that FABP9 protein is leaking out of STs into IF following cadmium chloride treatment. Quantification of the bands from all samples run from the cadmium chloride studies showed a highly significant increase (greater than 40 fold) in the amount of FABP9 in IF from animals treated with 3mg/kg $CdCl_2$. The graph also suggests an increase in FABP9 in IF from animals treated with 1.75mg/kg $CdCl_2$ although this did not reach statistical significance.

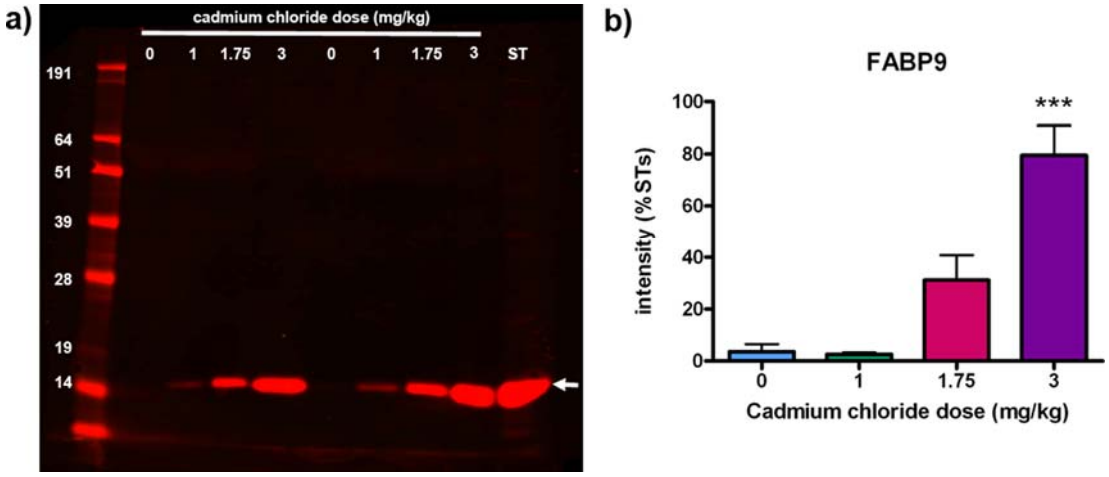


Figure 7-12 Representative Western blot probed with FABP9 antibody, showing interstitial fluid samples from control rats and animals treated with 1, 1.75 or 3.0mg/kg $CdCl_2$ and control homogenised seminiferous tubules (ST) (a). Arrow indicates FABP9 protein. (b) Graph showing quantification of 15kDa band (mean with SEM), thought to be FABP9 with values taken from samples run on four separate Western blots (n=7 samples for control, n=5 for 1mg/kg group, n=8 for 1.75 and 3mg/kg groups), expressed as a percentage of STs. Statistical analysis, one-way ANOVA followed by Tukey post-test. *** $p<0.001$ compared to control samples.

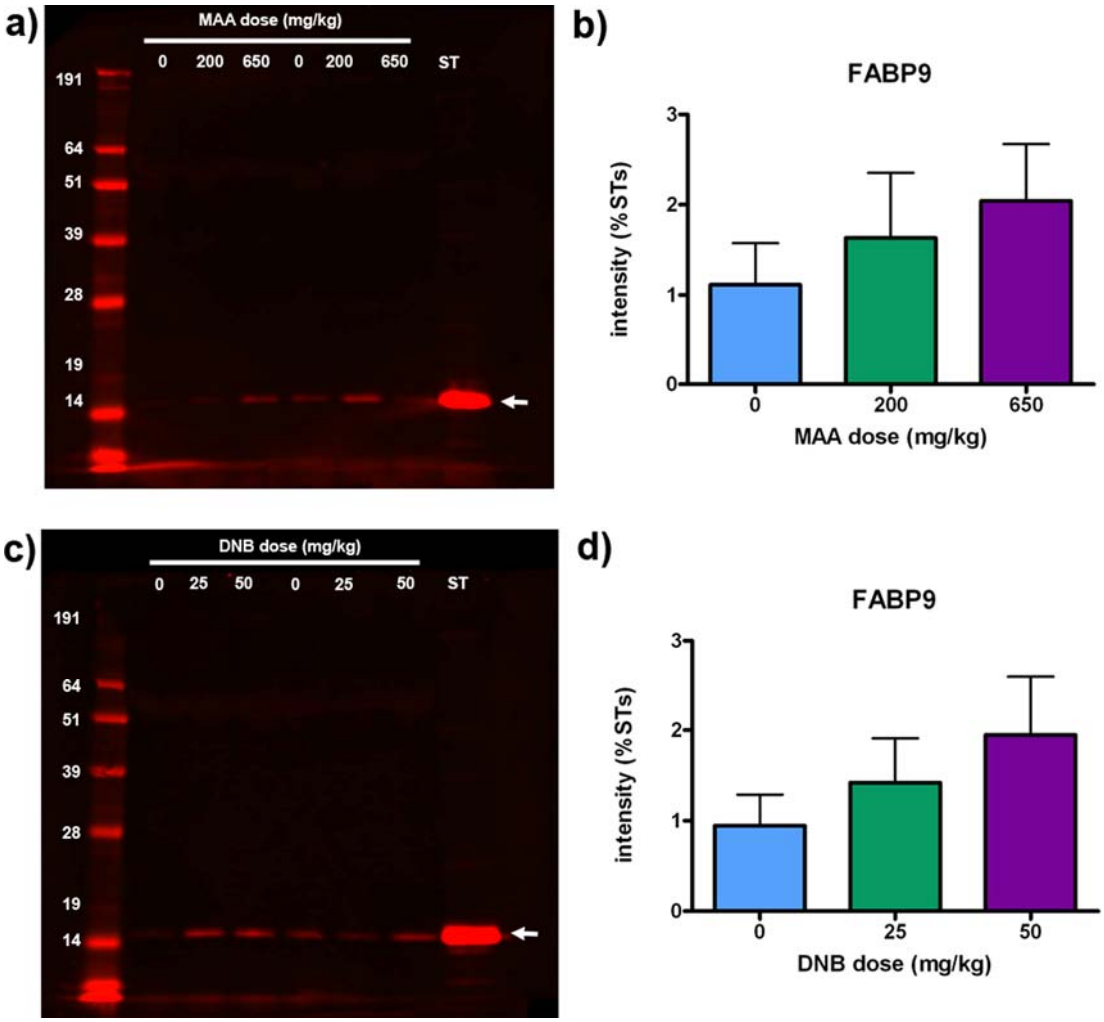


Figure 7-13 Representative Western Blots probed with FABP9 antibody, showing (a) interstitial fluid samples from control rats and animals treated with 200 or 650mg/kg MAA and control homogenised seminiferous tubules (ST), and (c) interstitial fluid samples from control rats and animals treated with 25 or 50mg/kg DNB and control homogenised seminiferous tubules (ST). Arrows indicate FABP9 protein bands. Graphs showing quantification of the 15kDa band (mean with SEM), thought to be FABP9 with values taken from samples run on three separate Western blots (n= 6 samples/dose group), expressed as a percentage of STs, for (b) MAA samples, and (d) DNB samples. Statistical analysis, one-way ANOVA followed by Tukey post-test showed no significant differences

Western blots with the FABP9 antibody were also run with IF samples collected from MAA and DNB studies (Figure 7-13). Bands were clearly seen at about 15kDa in size. The most prominent band on both Western blots was in the ST sample. Faint bands were detected with some of the IF samples from both MAA and DNB studies. Quantification of the intensity of the protein bands across two Western blots for each

treatment showed a trend for a slight increase in the amount of FABP9 in the IF samples across the dose range for both toxicants, although this was not significantly different from controls with any dose of either MAA or DNB treatment. The magnitude of the change is put in perspective by comparison with the levels of FABP9 detected after cadmium chloride treatment (Figure 7-12).

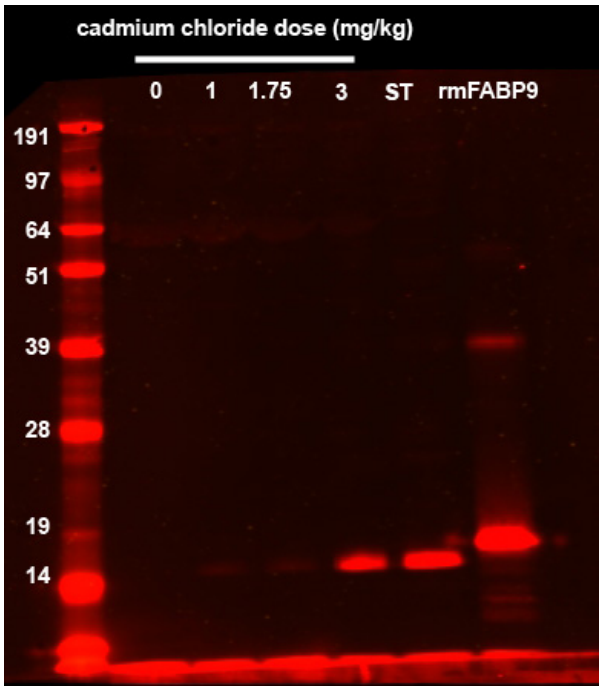


Figure 7-14 Western Blot probed with FABP9 antibody, showing interstitial fluid samples from control rats and animals treated with 1, 1.75 or 3.0mg/kg $CdCl_2$, control homogenised seminiferous tubules (ST), and recombinant mouse FABP9 protein (rmFABP9).

In order to confirm that the protein bands seen in the Western blots with the FABP9 antibody were actually FABP9, recombinant FABP9 protein was purchased. The recombinant protein was run on a Western blot with the FABP9 antibody and alongside IF samples from cadmium chloride treated animals and seminiferous tubules (Figure 7-14). Protein bands were detected in the IF sample from the 3mg/kg $CdCl_2$ and ST sample as well as the recombinant protein. The recombinant protein appeared to be slightly larger in size than the bands in the IF and ST sample, but this

is likely due to the way the protein was purified and the extra molecular weight could be due to the remains of the histidine tag. The larger band, at about 39kDa, also detected in the lane with the recombinant protein, could be the protein with the full tag for purification still attached. This blot suggests that the protein detected by the antibody in the IF and ST samples is FABP9. A further Western blot could be run, using the recombinant protein to block the FABP9 antibody, and look for the absence of the bands, but there was not enough recombinant protein available to do this.

7.3.5.2 FABP9 Gene Expression

Differences in FABP9 gene expression were also investigated in testis samples collected from the cadmium chloride, MAA, and DNB studies by TaqMan® PCR (Figure 7-15). The results showed no differences in expression of the FABP9 gene across the dose groups for all three toxicants, even though an effect on the protein was suggested with the higher doses in Figure 7-11. However, the protein expression of FABP9 has only been analysed qualitatively using immunohistochemistry and the staining observed is not very distinct. It could be that there is no change in the total amount of FABP9 protein in the testis following any treatment, or perhaps the gene expression studies were insensitive to any changes in gene expression.

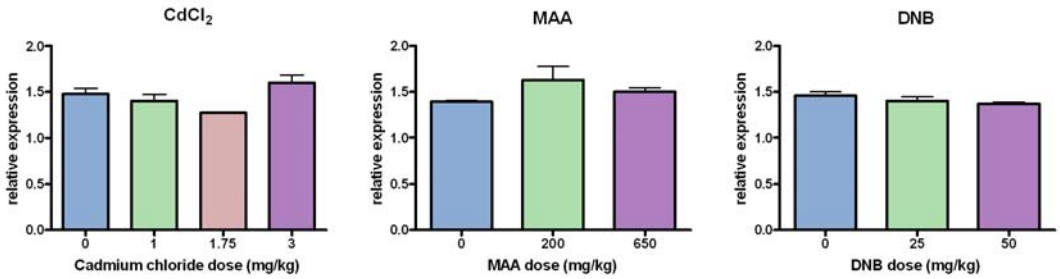


Figure 7-15 TaqMan® analysis of FABP9 gene in testis samples collected from animals treated with (a) cadmium chloride, (b) MAA, (c) DNB (mean with SEM). Expression relative to GAPDH expression. n=4 samples/dose group, except 3mg/kg $CdCl_2$ where n=3. Statistical analysis, one-way ANOVA followed by Tukey post-test showed no significant differences.

7.3.5.3 Further FABP9 Investigations

From the results seen with the three toxicant treatments, cadmium chloride, MAA and DNB, FABP9 seems to be a good indicator of protein leakage, as well as a potential biomarker candidate. Investigations with FABP9 were, therefore, carried out with samples from the glycerol, TGF-β3, and testis development studies.

Immunohistochemistry with the FABP9 antibody was carried out with control and treated tissue from the glycerol and TGF-β3 studies (Figure 7-16). Again, the staining was not distinct, but FABP9 was detected in spermatids in the control tissue, and similarly in the areas of the treated testes that did not seem to be affected by treatment (Figure 7-16c, f). In the areas that were affected by treatment, there appeared to be less staining for FABP9 (Figure 7-16b, e).

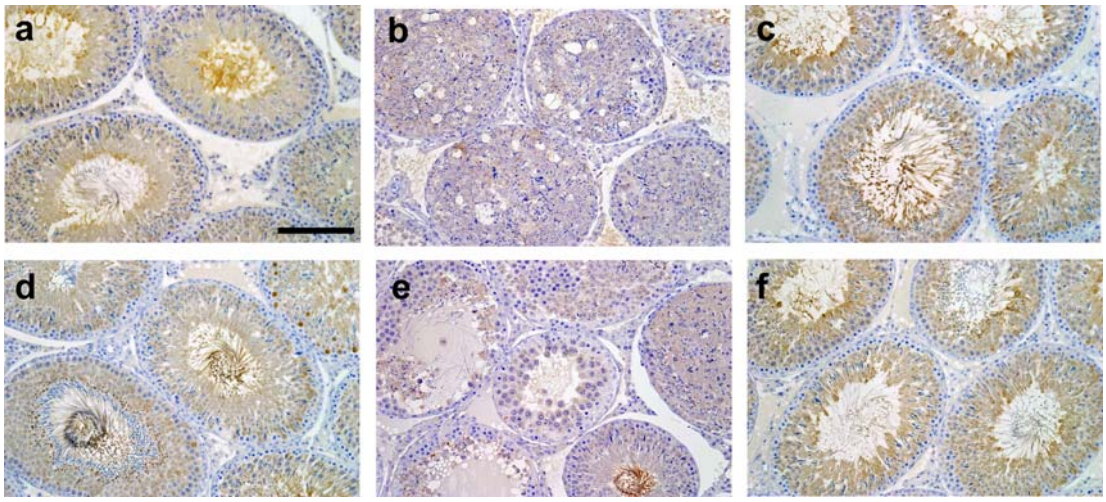


Figure 7-16 Testis sections from control rats (a, d) and rats treated 48 hours earlier with 15% glycerol (b,c) or 200ng TGF-β3 (e,f) immunostained to show FABP9 (brown) and counterstained with haematoxylin (blue). Scale bar represents 100μm.

Interstitial fluid samples collected from control testes and testes injected with either glycerol or TGF-β3 were run on Western blots with the FABP9 antibody (Figure 7-17). A large bright band was detected in the ST sample on both blots, showing that the Western blot had worked. Faint bands were evident in most IF samples from both the glycerol and TGF-β3 studies. Quantification of the bands showed a significantly

higher concentration of FABP9 in IF from testes injected with glycerol, compared to control testes, suggesting leakage from STs. No significant difference was observed between the control IF and IF from TGF-β3 treated testes.

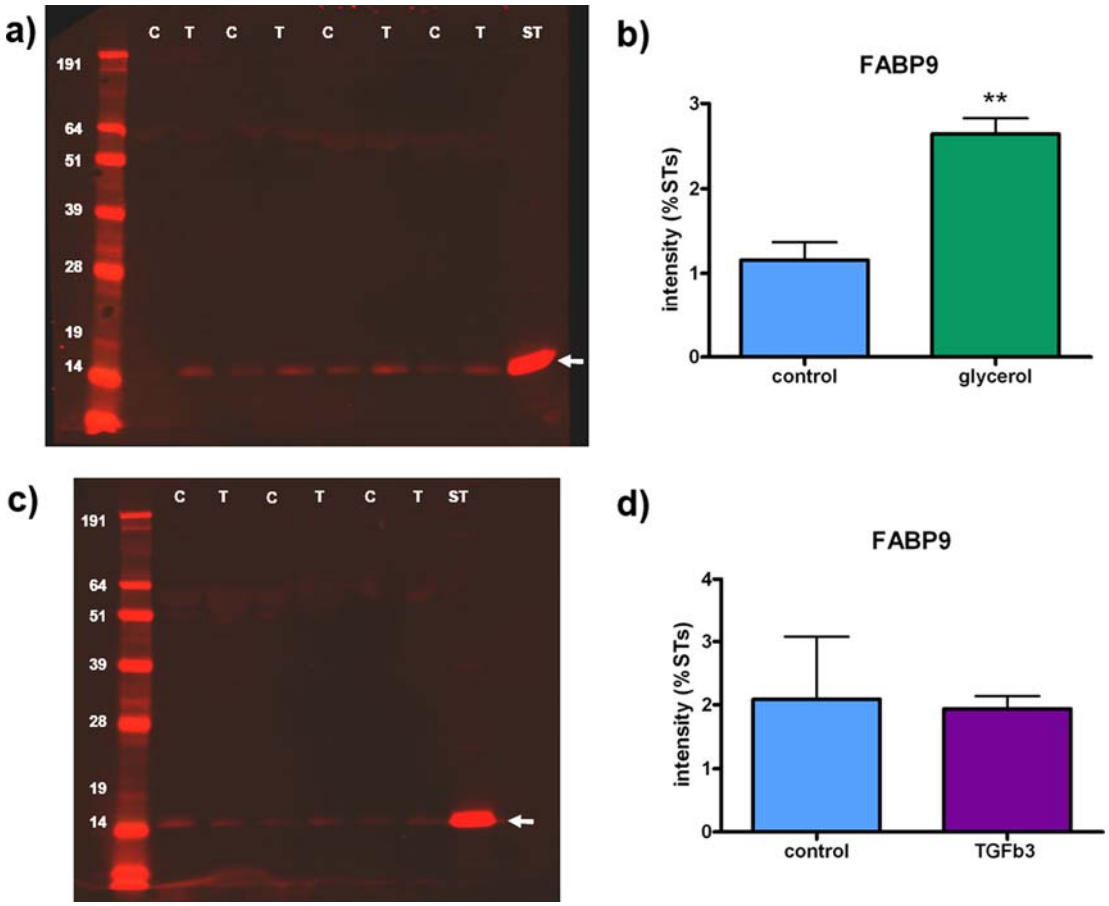


Figure 7-17 Western blots probed with FABP9 antibody, showing (a) interstitial fluid samples from control rats (C) and animals treated with 15% glycerol (T) and control homogenised seminiferous tubules (ST), and (c) interstitial fluid samples from control rats (C) and animals treated with 200ng TGF-β3 (T) and control homogenised seminiferous tubules (ST). Arrows indicate FABP9 protein bands. Graphs showing quantification of 15kDa band (mean with SEM), thought to be FABP9 expressed as a percentage of STs, with values taken from samples run on one Western blots for (b) glycerol samples (n=4 samples/dose group), and (d) TGF-β3 samples (n=3 samples/dose group). Statistical analysis, t-test. ** $p<0.01$ compared to control samples.

Testis sections collected as part of the testis development study were also stained to show FABP9 protein by immunohistochemistry (Figure 7-18). The results clearly showed that FABP9 is not present in the developing testis until day 25, where it

localised to spermatocytes. As shown previously in adult control tissue, FABP9 is also present in developing spermatids, but this stage is not present at day 25.

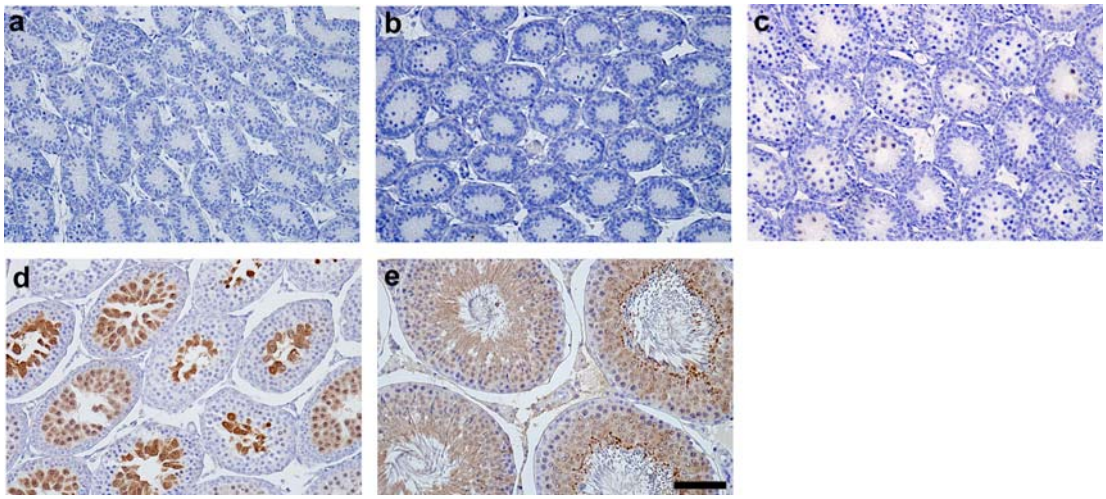


Figure 7-18 Testis sections from rats at a) day 10, b) day 15, c) day 18, d) day 25 and e) adult, immunostained to show FABP9 (brown) and counterstained with haematoxylin (blue). Scale bar represents 100µm.

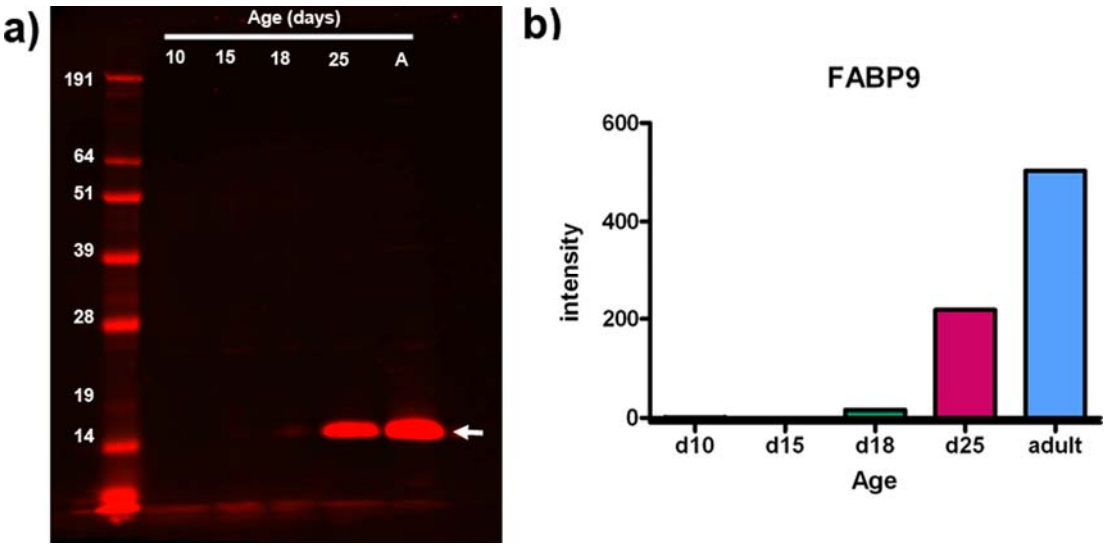


Figure 7-19 Western blot probed with FABP9 antibody (a), showing seminiferous tubule samples from day 10, 15, 18, 25 and adult (A) rats. Arrow indicates FABP9 protein band quantified. (b) Graph showing quantification of 15kDa band, thought to be FABP9 (mean with SEM) (n=1 sample/age).

A Western blot showing isolated seminiferous tubules from the different stages of testis development with the antibody to FABP9 is shown in Figure 7-19. The blot supports the immunohistochemistry results and shows the presence of FABP9 in STs at day 25 and in adult testis tissue, with a small amount present at day 18.

mRNA samples isolated from testes at the different stages of development were run with TaqMan® PCR to look at expression of the FABP9 gene (Figure 7-20). The results showed significantly more FABP9 expression at day 18, compared to day 10 and day 15. A significant increase in expression was also noted at day 25 (with similar levels to adult tissue) compared to days 10, 15, and 18. This supports the protein immunohistochemistry and Western blot data.

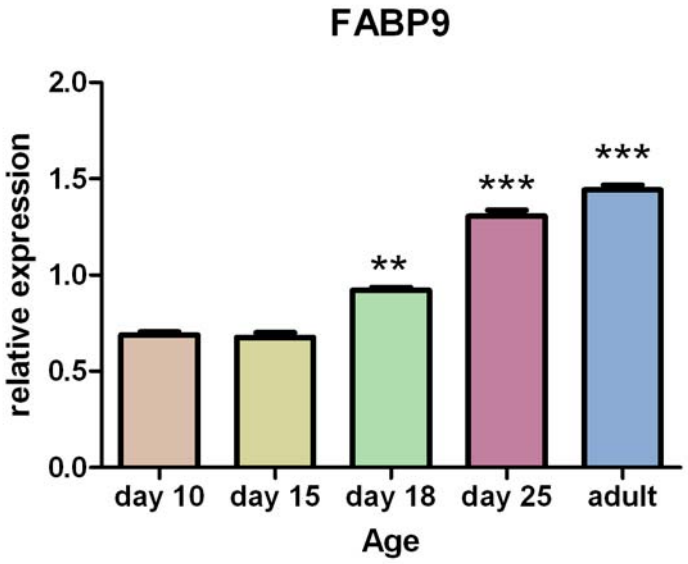


Figure 7-20 TaqMan® analysis of FABP9 gene in testis samples collected from days 10, 15, 18, 25 and control adult rats. Expression relative to GAPDH expression. n=4 samples/age except day 15 where n=2 and adult where n=12. Statistical analysis, one-way ANOVA followed by Tukey post-test. ** $p<0.01$, *** $p<0.001$ compared to day 10 and day 15 samples (and day 18 samples versus day 25 and adult).

7.4 Discussion

Biomarkers for testicular toxicity would be useful for assessment of damage in regulatory toxicology studies in the pharmaceutical industry. They would allow detection of testicular damage at various time points throughout the study rather than just at the end. This would give a profile of the damage over the study period, and also a chance to detect damage much earlier in a study. This could lead to a reduction in the number of such studies needed as a compound causing testicular damage could be screened out at an earlier stage than might be the case with current methods of testicular damage detection.

Five potential candidate proteins were investigated for their suitability as biomarkers for testicular toxicity. DAZL, a well-studied germ cell marker, is small in size at 33kDa, and is specifically expressed in spermatocytes. Immunohistochemical analysis suggested a dose dependent decrease in DAZL protein following MAA and DNB treatment. This dose-dependence would be ideal for a biomarker, as the concentration of biomarker detected would correlate to the extent of testicular damage. However, Western blotting with a DAZL antibody to investigate the presence of the protein in IF samples following toxicant treatment, suggested that DAZL does not leak out of STs into IF following high dose $CdCl_2$ treatment or after MAA or DNB treatment, although there was some discrepancy in the size of the bands identified on the Western blots compared to the predicted size. If DAZL protein does not leak out of STs into IF following BTB damage, it could not be used as a biomarker.

VASA is another well-studied germ cell protein. It is larger than DAZL at 76kDa in size and therefore was expected to be less likely to leak out of the STs. Immunohistochemical analysis of VASA protein showed that it was present in spermatocytes and spermatids, and that there was an apparent dose-related reduction in the amount of protein present in the testis following treatment with MAA, DNB and $CdCl_2$. Western blot analysis of VASA protein in IF samples collected from the three toxicant studies, showed the presence of VASA protein in ST samples, and IF

from animals treated with 3mg/kg $CdCl_2$, with a small amount present in IF from animals treated with 1.75mg/kg $CdCl_2$. This suggests that VASA protein is leaking out of STs into IF following mid and high dose cadmium chloride treatment in a dose dependent manner. This supports the results presented in chapter 5, where it was found that proteins only leak from STs into IF following $CdCl_2$ induced damage to the BTB, and not following spermatocyte loss with MAA treatment or Sertoli cell damage with DNB treatment. The apparent leakage of VASA disagrees with the hypothesis that small molecular weight proteins are more likely to leak out of STs into IF, as VASA protein is a relatively large protein.

DAZL and VASA are both germ cell derived proteins, however, only VASA appeared to leak from the seminiferous tubules into the IF following high dose cadmium chloride treatment. This difference could be due to the differences in localisation or function of the proteins. A further explanation for the difference could be related to the size discrepancy with the protein bands observed on the Western blots with the DAZL antibody and the predicted molecular weight of DAZL. The proteins observed could contain some of the epitopes recognised by the DAZL antibody but not actually be DAZL protein. If this were the case, perhaps the effects observed are not related to DAZL protein.

ADAM3 is a larger molecular weight protein (110kDa precursor protein) found in spermatids. Initial investigations with ADAM3 suggested that the antibody was not very sensitive, as high concentrations were required for successful immunohistochemistry. Western blot analysis of IF samples from control and high dose cadmium chloride treated animals did not appear to detect ADAM3 in IF samples and detected bands of unexpected size in the ST sample. For these reasons, ADAM3 was not investigated further as a potential biomarker.

Calpastatin is another relatively large protein, 76kDa in size, expressed in spermatids. A reduction in calpastatin protein was suggested by immunohistochemistry following high dose treatment of all three testicular toxicants,

although the lack of effect following low dose treatments does not make calpastatin an ideal biomarker candidate. Initial Western blot investigations with IF from a control animal and an animal treated with 3mg/kg $CdCl_2$, and control STs, showed a range of different sized protein bands detected by the calpastatin antibody. None of these bands corresponded to the protein bands suggested by the antibody manufacturer. The Western blot was repeated using a different electrophoresis system for initial separation of protein bands (tris-glycine gel system) to investigate whether the original gel system used was affecting the way the proteins ran. The same protein bands were detected with the second system (results not presented), validating the original system used but providing no further explanation for the unexpected sizes of the protein bands. A calpastatin blocking peptide was not available to investigate whether the bands seen are really calpastatin protein. To further investigate this, an antibody to somatic calpastatin could be used to probe a Western blot and see whether bands of the same size are detected by probing for the conserved region of the somatic and testis calpastatins. An appropriate antibody was not available so calpastatin was not investigated further as a potential biomarker candidate.

The final potential biomarker candidate investigated was FABP9, a small protein (15kDa) found in spermatocytes and spermatids. The presence of FABP9 protein in IF samples following $CdCl_2$, MAA and DNB treatment was investigated by Western blotting. FABP9 protein was detected in IF samples from animals treated with mid and high dose $CdCl_2$ in a dose dependent manner. No significant differences were found with FABP9 in IF from all doses of the MAA and DNB studies. This suggests FABP9 is leaking out of STs into IF following cadmium chloride treatment and supporting the results described in chapter 5. The identity of the protein seen on the Western blots was confirmed using a recombinant FABP9 protein. TaqMan® PCR showed no change in FABP9 gene expression. However, the protein analyses considered the quantification of FABP9 protein leaking into the IF, rather than the total amount of FABP9 protein present in the testis, so this gene expression data does not oppose the protein data. FABP9 is a promising biomarker protein, due to its

small size, leakage from STs into IF following BTB damage and strong detection by Western blotting. Investigations with tissue from the glycerol, TGF-β3 and development studies and FABP9 were also carried out.

Immunohistochemistry to show FABP9 in tissue collected from glycerol and TGF-β3 treated testes, suggested a reduction in FABP9 expression in the localised areas of treatment induced damage. Western blot analysis of FABP9 in IF samples collected following these treatments, showed significantly more FABP9 protein in IF from glycerol treated testes, compared to control, even though the treatment had only a localised effect on the testis. This suggests that FABP9 could be a sensitive marker, as changes in amount of protein in IF following slight damage can be detected. No changes were detected in the concentration of FABP9 in IF from TGF-β3 treated or control testes. This could be due to the very small, localised amount of damage caused by the TGF-β3 treatment, and therefore no protein leakage from STs into IF. These results support those described in chapter 6.

Testis sections from day 10, 15, 18, and 25 were immunostained to show FABP9. The results clearly show that FABP9 is present in the spermatocytes at day 25, but no FABP9 is present at or before day 18, presumably because spermatocytes of the right stage of development are not present at these ages. FABP9 is thought to have a role in the wave of apoptosis at this stage in development (Kido and Namiki, 2000), which supports the presence of FABP9 at day 25. Isolated seminiferous tubules run on a Western blot with the FABP9 antibody also support this result, where FABP9 was detected in small amounts at day 18, with an increase at day 25 to about half the concentration of FABP9 present in adult STs. TaqMan® analysis showed that the FABP9 gene was expressed in significantly higher amounts at day 18, compared to days 10 and 15, and expression increased to the same level as in adults at day 25. This supports the protein data.

Following these investigations, it is concluded that FABP9 and VASA are both promising biomarker candidates for detection of damage to the BTB. If these were to

be developed for use in regulatory toxicology studies, a sensitive assay would need to be developed. Interstitial fluid samples have been used for protein analysis in these investigations, but the protein biomarker would need to be detectable in blood samples for use in toxicology studies, where the biomarker will be much more dilute. Although the FABP9 antibody appears to be quite sensitive for detection of differences in IF protein levels, it probably would not be sensitive enough for detection of low levels in blood samples. ELISAs could be investigated for analysis of VASA and FABP9 in blood samples, or more sensitive detection methods such as FACTT (fluorescent amplification catalysed by T7 polymerase technique) (Zhang et al., 2006) could be evaluated.

Following the results presented in chapters 5 and 6, these proteins would, however, only be of use in detection of damage that involved disruption of the blood-testis barrier, rather than general disruption to spermatogenesis. Therefore, such a marker would probably only be useful if you were specifically looking for damage to the blood-testis barrier, and would not be useful in drug toxicology testing, where the site and mechanisms of toxicity are unlikely to be known.

8 Applications and Refinements for Detection of Testicular Toxicity

8.1 Introduction

The investigations into proteins present in interstitial fluid (IF) using Western blotting and Coomassie stained gels throughout this thesis all illustrate one limitation of this approach; the large amount of albumin protein in the samples, which could be masking other proteins. A study was carried out to investigate three commercially available kits designed for removal of albumin from samples, and their ability to remove albumin from IF and blood samples collected during the cadmium chloride studies, as well as whether the other proteins in the samples were affected during the process.

Three kits were chosen due to their differing mechanisms of action. The BioRad Aurum™ Affi-Gel® Blue Mini Kits and Columns utilise the Cibacron Blue F3GA dye mechanism for albumin removal. The kit is designed for the removal of albumin from serum or plasma samples using single use spin down columns. The second kit, ProteoExtract® Albumin Removal Kit from Calbiochem, uses a novel albumin specific resin in affinity gravity flow columns. It claims to provide a fast, reproducible and highly specific method to remove albumin from samples such as plasma, serum, or cerebrospinal fluid, and to have efficiency with samples from a range of species (human, rabbit, rat, mouse, pig). The final kit chosen was the Qiagen Qproteome™ Murine Albumin Depletion Kit. This kit again uses affinity columns but with monoclonal antibodies that bind albumins, in the form of single use spin down columns. This kit is specifically designed to deplete albumin in mouse or rat plasma and serum samples.

IF and peripheral vein blood plasma samples from animals treated with 1.75mg/kg $CdCl_2$, and control animals (and for one kit from animals treated with 0.2mg/kg $CdCl_2$), were run through the kits and ‘before’ and ‘after’ samples were then run on

Coomassie stained gels to assess the reduction of albumin, and whether any other proteins had been removed in the process. IF samples were used because they have been the main sample used for the study of proteins in this thesis. Peripheral vein blood was also collected from early cadmium studies for use in investigating whether proteins leak into the blood from IF, but were not investigated in the scope of this idea. Samples from animals treated with 1.75mg/kg $CdCl_2$ were included, because protein leakage from STs to IF has previously been observed with this dose, allowing an evaluation of the effect of the kits on proteins of interest. Samples from the 3mg/kg dose group were not available.

Removal of albumin from samples would be a useful refinement to the analysis of proteins in IF and blood samples, but this approach will not be refined completely, and the investigations in this thesis suggest that this is not a suitable way to detect testicular damage. A different approach was investigated, which although will not provide an early warning detection system for testicular toxicity, may improve the detection of testicular damage at the end of a study.

Automated immunohistochemistry was carried out on testis sections from animals treated with $CdCl_2$, MAA or DNB, staining for the germ cell protein DAZL, as this had been shown earlier to be a good, clean marker for germ cells. Stained sections were analysed using Image Analysis software, and the percentage area of DAZL staining calculated for each section, as a means of quantifying germ cell area.

8.2 Methods

8.2.1 Albumin Removal Study

A small study was set up to look at the removal of albumin from interstitial fluid samples and peripheral blood plasma samples from control and cadmium chloride treated rats.

8.2.1.1 Bio-Rad Kit – Aurum™ Affi-Gel® Blue Mini Kit

Samples were run through the kit (Bio-Rad, Herts, UK) according to the manufacturers' instructions. 20mM Tris buffer, pH8.3 was chosen as a suitable low-salt application buffer. The columns were placed in test tubes to allow the resin to settle, then the residual buffer was drained by gravity flow. The columns were washed twice with Tris buffer, and the resin bed dried by centrifugation. 75µL of each sample was diluted with 225µL Tris buffer. Dried columns were placed in clean collection tubes. 300µL of sample was loaded onto the top of the column and allowed to drain through by gravity-flow. Columns were then centrifuged at 10,000x*g* for 20 seconds before washing the resin with 400µL Tris buffer, followed by a final centrifuge at 10,000x*g* for 20 seconds. The albumin depleted sample in the collection tube was stored at -20°C.

8.2.1.2 Calbiochem Kit – ProteoExtract® Albumin Removal Kit

Samples were run through the kit (Calbiochem, Merck Biosciences, Nottingham, UK) according to the manufacturers' instructions. Samples were diluted 10 fold with the supplied ProteoExtract® Albumin Binding Buffer (35µL sample with 315µL buffer). Storage buffer was removed from the columns and 1mL Albumin Binding buffer was added to equilibrate the columns. Columns were then placed in clean collection tubes, the diluted samples added and allowed to pass through the column by gravity-flow. 600µL buffer was added to wash the columns and allowed to pass through by gravity-flow. This step was repeated to thoroughly wash the column. The resulting albumin depleted samples in the collection tubes were stored at -20°C.

8.2.1.3 Qiagen Kit – Qproteome™ Albumin/IgG Depletion Kit

Samples were run through the kit (Qiagen, West Sussex, UK) according to the manufacturers' instructions. PBS was used as the dilution buffer. Samples (25µL) were diluted with 75µL PBS. Qproteome Depletion Spin Columns were briefly centrifuged to remove resin from the cap, caps and bottom closures were removed,

and storage buffer was drained out by gravity-flow. Columns were equilibrated with 2x 0.5mL aliquots of PBS, each aliquot being allowed to run out by gravity-flow. The bottoms of the columns were sealed with a luer plug, and the diluted sample added. Columns were shaken to form a homogenous suspension and incubated on an end-over-end shaker at room temperature. Luer plugs were removed and columns transferred to clean collection tubes. The flow-through was collected by centrifugation at 500x*g* for 10 seconds. Columns were washed with 2x 100µL aliquots of PBS, collecting the flow-through by centrifugation at 500x*g* for 10s each time. The resulting combined flow-throughs containing the albumin depleted samples were stored at -20^{o}C.

8.2.1.4 Protein Assay

Albumin depleted samples from all three kits were assayed for protein content using the Bio-Rad DC assay (section 3.7). Samples were diluted as necessary in the appropriate buffer used for the albumin depletion. BSA standards were also prepared in each buffer, and separate standard curves made for samples from each kit to ensure accuracy of results.

8.2.1.5 Analysis of Albumin-depleted Samples using 1D Gels

Albumin depleted samples were run alongside corresponding original samples on 1D gels, and stained for total protein (sections 3.8 and 3.9). Protein bands were analysed using ImageQuant software (section 3.9.1). The amount of each protein present in the albumin depleted sample was expressed as a percentage of the amount of protein in the original sample (100% indicates no loss of protein in sample after running through albumin depletion kit, less than 100% indicates some loss of the protein, and greater than 100% indicates a gain of protein). In order to obtain more accurate results, each sample was run on three separate gels, and the average percentage change for each sample calculated.

8.2.2 Automated Immunohistochemistry

Fixed tissue was processed and sectioned as described in section 3.4.2.1. As with the bench protocol for DAZL immunohistochemistry, the slides were first dewaxed and rehydrated (section 3.5.1), before carrying out antigen retrieval in citrate pH6 buffer (section 3.5.2). The slides were then loaded onto the Bond System (Vision Biosystems, Leica, Bucks, UK), and run with the Bond Polymer Refine detection kit, which includes a peroxidase block, post-primary polymers and DAB chromagen. DAZL, primary antibody was diluted to 1:1000 in antibody diluent supplied with the kit, and loaded onto the machine. The program was set to exclude the haematoxylin counterstain step for ease of image analysis, and the machine was left to run overnight. When the program finishes, the Bond machine washes the slides with water every 5 minutes to ensure they do not dry out. Slides were removed from the machine, dehydrated and fixed with coverslips using Pertex (section 3.5.8).

8.2.3 Image Analysis

Image analysis was carried out using a Nikon Eclipse E800 microscope with camera attached, and Image-Pro 6.2 software. Sections were first tiled using the camera to create a picture of the whole section on the computer screen (Figure 8-1a). The total area of the section was calculated by drawing round the section. The threshold for staining to be included in the analysis was set by selecting two or three of the darkest coloured pixels. The software then selected all pixels above the threshold in the tissue section, allowing the total area of most intense DAZL staining to be calculated (Figure 8-1b). The percentage of DAZL staining was calculated for each section and graphed using GraphPad Prism. Statistical analysis was carried out using a one way ANOVA, followed by Tukey's post-test.

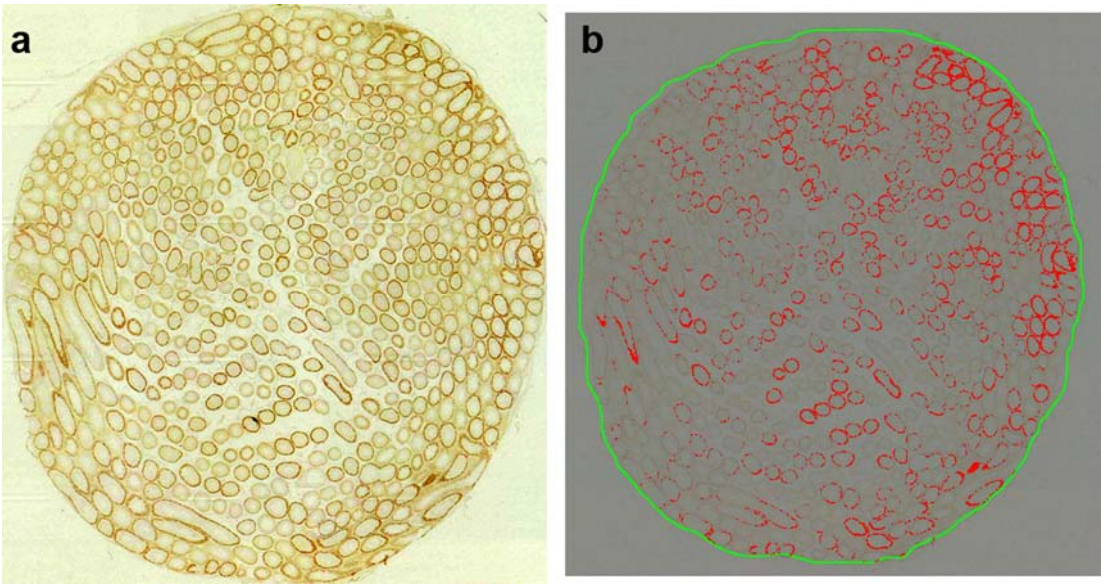


Figure 8-1 Testis section stained for DAZL using automated immunohistochemistry, photographed using Image Analysis software (a). The area of the section was calculated (b-green line) and the area of staining then calculated (b – red).

8.3 Results

8.3.1 Albumin Removal from Samples

Coomassie gels showing IF and blood samples before and after running through the albumin removal kits are shown in Figure 8-2. Albumin protein can be seen as the band at 64kDa. There appears to be a decrease in the amount of albumin in most of the samples run in all the kits (except samples IF4 and IF7 which don't seem to have changed much). This suggests success of the albumin depletion kits. However, not all the albumin was depleted. This perhaps would not be necessary as the decrease in size of albumin protein band does make it easier to see other protein bands of a similar size on the Coomassie gels. The percentage change in the amount of albumin in samples before, compared to after, running through the kit was calculated for each sample. Samples were run on three separate gels and the average percentage change calculated. The results are shown in Figure 8-3.

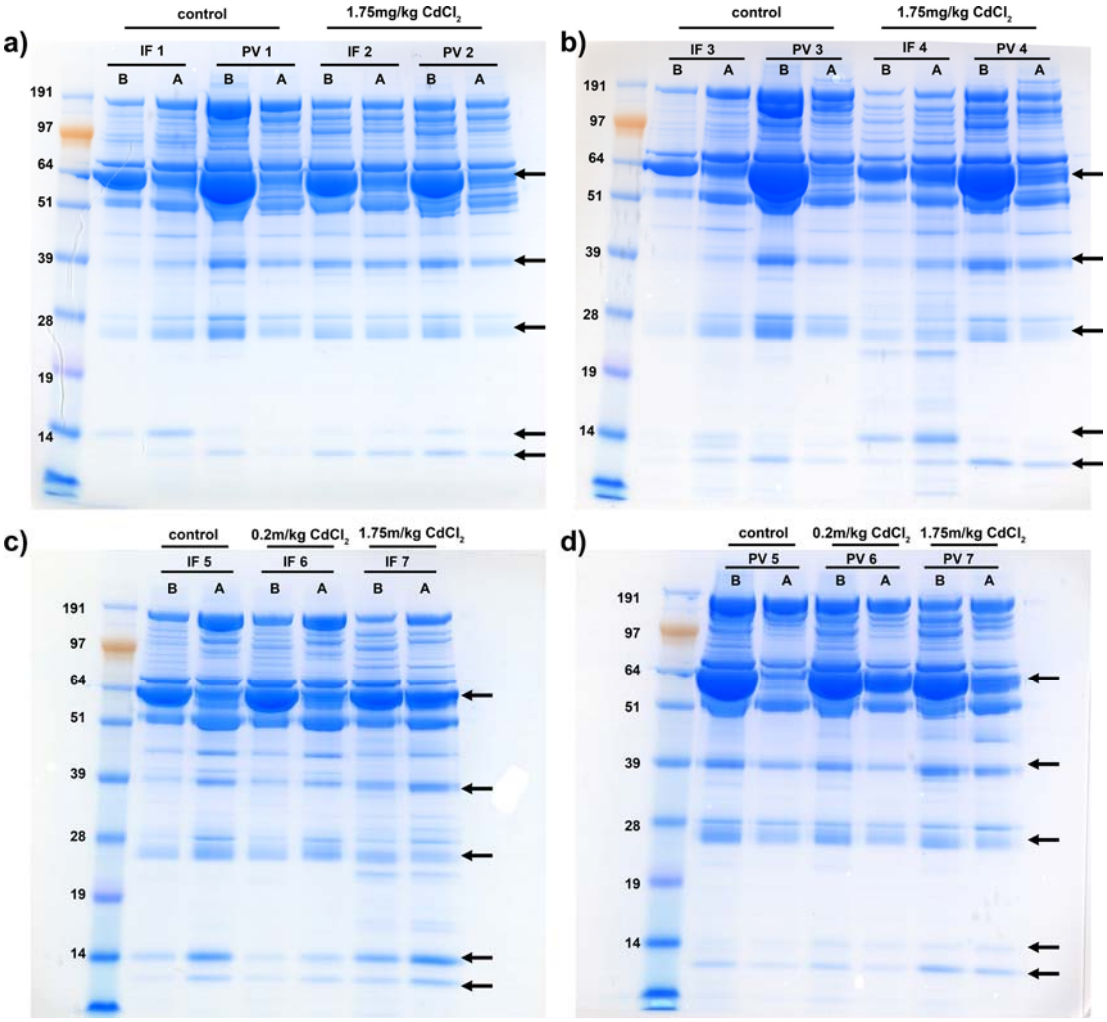


Figure 8-2 Coomassie stained gels showing samples before (lanes labelled B) and after (lanes labelled A) they were run through albumin removal kits from Qiagen (a), Calbiochem (b) and BioRad (c, d). IF – interstitial fluid, PV – peripheral vein blood. Different samples were used for each kit. Arrows indicate protein bands quantified (shown in Figure 8-3 and Figure 8-4).

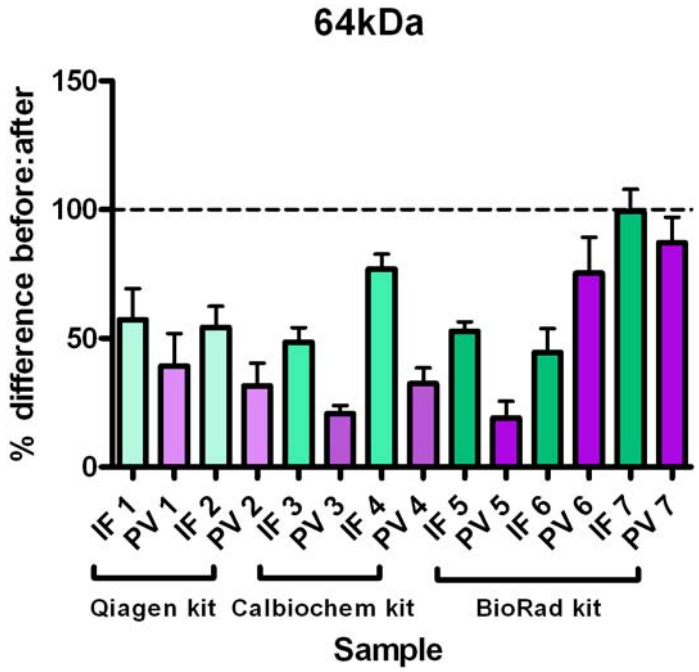


Figure 8-3 Graph showing percentage change in albumin (64kDa band) after samples were run through albumin removal kits compared to before for all samples (mean with SEM). N=3 for each sample. Dashed line at 100% indicates where the same amount of albumin was present before and after albumin depletion.

This quantification showed a decrease in albumin in all samples following running through the albumin depletion kits except for sample IF7, in which the amount of albumin did not change. Figure 8-3 shows that there was considerable variation in the amount of albumin that was removed from different samples, and all of the kits were slightly better at removing albumin from the blood plasma samples than from the IF samples. This could be for two reasons. Firstly, there was a larger amount of albumin in the initial blood samples than in the IF samples (seen on the gels in Figure 8-2), and therefore more chance for the kits to remove more albumin. Secondly, the kits are designed to remove albumin from blood plasma samples rather than from interstitial fluid samples, as blood samples are a more widely used as a test material. Perhaps this observation is therefore also more relevant.

The Coomassie stained gels (Figure 8-2) and graph (Figure 8-3) do not show any of the three kits tested to be consistently better than the others at removing albumin

from samples. Samples run through the BioRad kit had both the best and the worst change in albumin before and after running through the kit (PV5 compared to IF7). There was also a large difference between the best and worst sample change with the Calbiochem kit (PV3 compared to IF4). This suggests that perhaps the Qiagen kit is the better kit as it provided a more consistent amount of albumin depletion.

Another consideration when using albumin removal methods is whether the method removes proteins of interest from the samples along with the albumin. The percentage change after albumin removal of four proteins identified in all the samples (arrows, Figure 8-2) was calculated. From the gels, it is hard to tell whether the amounts of the proteins have increased or decreased following albumin depletion. Again the mean percentage change was calculated after running the samples on three separate gels, and the results are shown in Figure 8-4. It is important to note that because 1D gel electrophoresis was used to investigate the proteins present in samples, that the protein bands on the gels could contain more than one different protein of the same molecular weight. However, because the analysis involves a comparison of samples before and after albumin retrieval, the same proteins should be identified in the same bands.

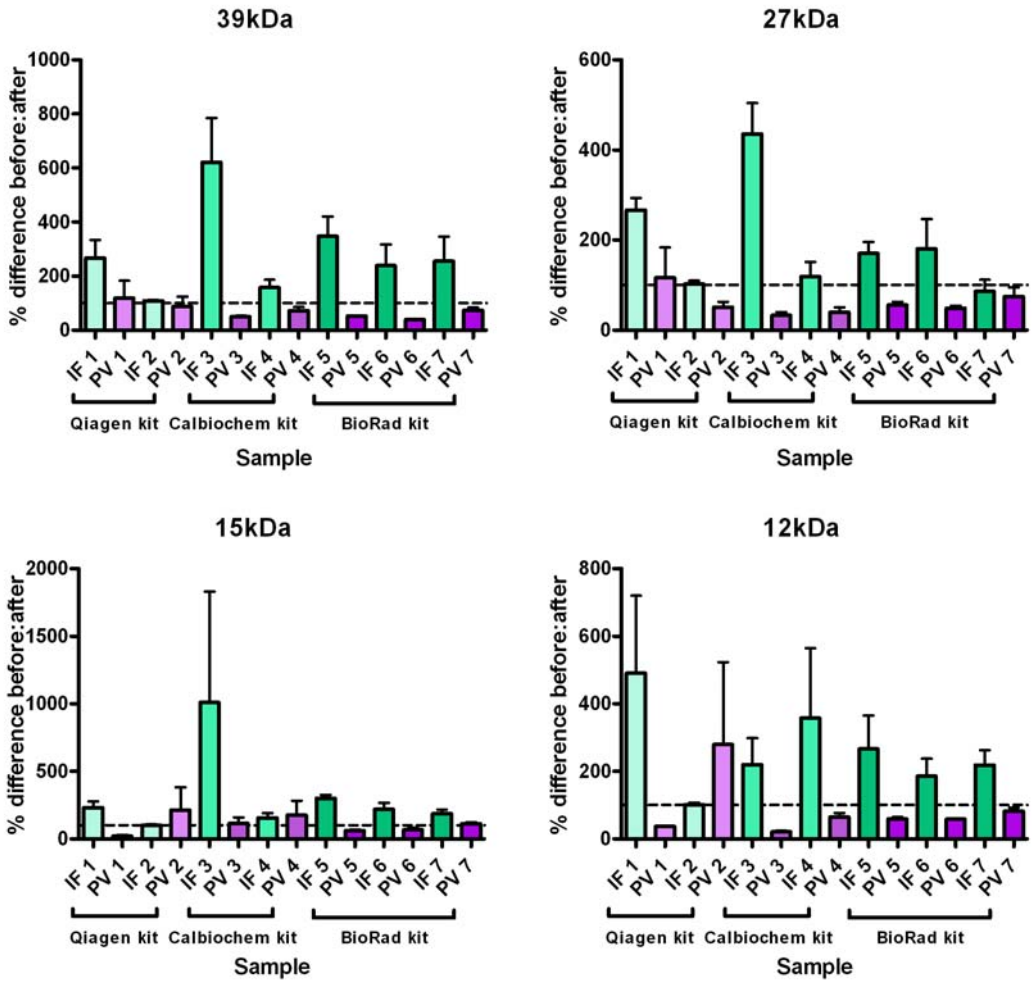


Figure 8-4 Graphs showing the percentage change after running samples through albumin removal kits for proteins of interest (indicated in Figure 8-2) (mean with SEM). N=3 for each sample. Dashed line at 100% indicates that the same amount of protein was present before and after albumin depletion.

The graphs in Figure 8-4 show a range of effects on the different proteins in the different samples. Some, such as the 39kDa protein in sample IF3, have increased in concentration following albumin removal. This could be because the same amount of total protein of each sample was loaded onto the gels, so as there is less albumin present in the sample, the concentration of the other proteins will increase, and more could have been loaded onto the gel in the 'after' sample. This, again, shows success of the albumin depletion and would improve detection of proteins of interest in the samples. To have any application, the amount of the proteins should not decrease

following albumin depletion, and in the majority of the IF samples, there appears to be the same amount or more of each of the proteins investigated with all three albumin removal kits. However, many of the blood plasma samples showed a decrease in the amount of the four proteins investigated, suggesting that some non-specific binding/depletion has occurred. This agrees with the results seen with albumin, which suggests that the kits are better at removing albumin from blood plasma samples, compared to IF samples, and are also better at binding non-specific proteins, which could be due to the kits being more tailored to blood samples than IF samples. However, the minimal number of wash/elution steps were carried out for each kit, and further steps could have been carried out with the kits which might have increased the percentage recovery of these proteins. From the loss of proteins of interest approach, there does not appear to be any advantage to any of the kits tested. If albumin removal were important for a study, albumin removal would need to be optimised for the samples collected.

8.3.2 Image Analysis: An Alternative Method for Detection of Testicular Damage

A different approach to assess testicular toxicity was investigated using automated immunohistochemistry and image analysis. DAZL staining was used as it is a germ cell marker and in previous chapters (chapter 5) was shown to be a good marker for damage to the testes caused by MAA and DNB treatments. The DAZL antibody also gave strong staining with low background which is essential for the semi-automated analysis to be feasible. Three or four sections were used for each dose of each treatment and the percentage of DAZL staining was calculated using Image-Pro software.

The results with testis sections from animals treated with various doses of cadmium chloride are shown in Figure 8-5. Significantly less staining was seen with testis sections from animals treated with 3mg/kg cadmium chloride compared to control or

lower dose groups. Considering the extensive damage to the seminiferous tubules, caused by 3mg/kg $CdCl_2$, this result was not surprising. However, a decrease in the amount of DAZL staining was not detected with the 1.75mg/kg $CdCl_2$ dose group, even though this dose has previously been shown to cause some damage to the seminiferous tubules (chapter 5). This could be due to the small numbers of sections investigated, or the subjective nature of the technique, where it is hard to ensure the same threshold of staining has been selected in each section. The 1.75mg/kg dose of $CdCl_2$ may also not have an effect on the number of DAZL positive germ cells present.

The results seen with sections from MAA treated animals are shown in Figure 8-6. A significant decrease in the percentage of DAZL staining can be seen with sections from animals treated with 650mg/kg MAA. This agrees with the treatment effects observed in chapter 5, and the nature of MAA, which causes selective depletion of pachytene spermatocytes.

In Figure 8-7, the results with tissue sections taken from animals treated with DNB are shown. There was a trend for less DAZL staining following treatment with 25mg/kg DNB compared to control tissue and a significant decrease in DAZL following treatment with 50mg/kg DNB. DNB treatment affects the Sertoli cells with a secondary loss of spermatocytes, so a decrease in DAZL staining would be expected following the high dose.

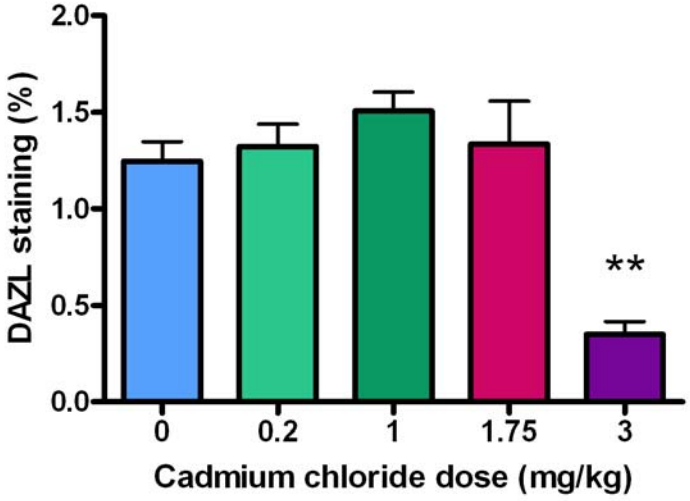


Figure 8-5 Graph showing percentage of DAZL staining in testis tissue sections from animals treated with 0, 0.2, 1, 1.75 or 3mg/kg cadmium chloride. (log transform results +1). n=3 samples/dose group, statistical analysis one-way ANOVA followed by Tukey post-test. ** p<0.01 compared to control samples.

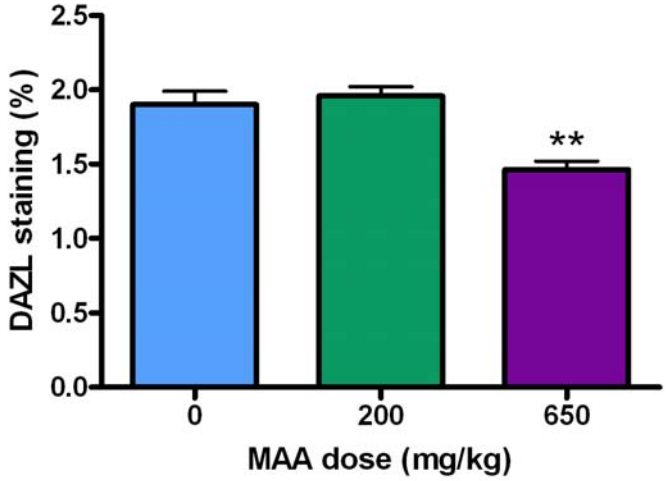


Figure 8-6 Graph showing percentage of DAZL staining in testis tissue sections from animals treated with 0, 200 or 650mg/kg MAA. (log transform results +1). n=4 samples/dose group, statistical analysis one-way ANOVA followed by Tukey post-test. ** represents p<0.01 compared to control samples.

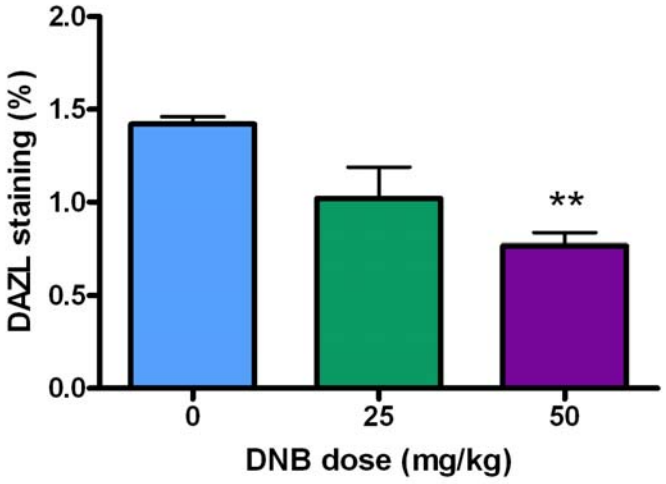


Figure 8-7 Graph showing percentage of DAZL staining in testis tissue sections from animals treated with 0, 25 or 50mg/kg DNB. (log transform results +1). n=4 samples/dose group, statistical analysis one-way ANOVA followed by Tukey post-test. ** represents $p<0.01$ compared to control samples.

8.4 Discussion

One of the problems with analysis of proteins in interstitial fluid samples throughout this thesis was the large amount of albumin present in the samples which limited the total amount of protein that could be loaded onto gels and, therefore, limited the sensitivity to detect low abundance proteins on the gels. Three commercially available kits for removal of albumin were investigated as a potential refinement for the analysis of such samples in future investigations. The kits all involved application of the sample to single use columns, but had different mechanisms of albumin removal (Table 8-1).

The Aurum™ Affi-Gel® Blue Mini Kit from BioRad utilises the Cibacron Blue F3GA dye mechanism for albumin removal. The kit was fairly easy to use, although the instructions state "do not overdry resin bed and frit" during one of the centrifugation steps, and it was hard to tell whether the columns had been overdried. The method was relatively quick to carry out, although buffer had to be prepared to run through the kit. A range of buffers are suggested in the instructions and if this kit

were to be used again, the buffer would need to be optimised for the samples. This, kit also required the largest sample volume (125µL is suggested), and it was hard to choose interstitial fluid samples with enough volume to run through the kit. This however, was not a problem for the blood plasma samples, and IF samples could have been diluted further if necessary. The albumin depletion of the samples run through this kit was very variable (ranging from 0.2 to 87.6% albumin depleted) and from the analysis of the four proteins of interest, there does seem to be some loss of other proteins during the process, possibly through non-specific binding. The lack of specificity of Cibacron dye based albumin removal has been previously described (Steel et al., 2003; Gianazza and Arnaud, 1982; Subramanian, 1984) and a published study comparing five commercially available albumin and IgG depletion kits (including the Calbiochem ProteoExtract kit, and the BioRad Aurum Serum Protein Minikit) also showed that the BioRad kit had the greatest non-specific binding, and the lowest reproducibility of the kits tested (Bjorhall et al., 2005). This publication investigated non-specific binding by analysing the proteins present in depleted serum samples, plus the elution of bound proteins from depletion columns, and running all samples diluted 1:100 on 1D-gels so that the percentage of proteins present in the depleted serum compared to original serum sample could be calculated. Perhaps it would have been better to have loaded the same volume of sample on the gels run in this study rather than the same amount of protein to show whether there was a reduction in the amount of protein of interest following albumin removal.

The second kit, ProteoExtract® Albumin Removal Kit from Calbiochem, uses a novel albumin specific resin in affinity gravity flow columns. The kit was quick and easy to use, with buffer supplied in the kit, and a simple gravity flow protocol. A small sample volume of 20-60µL can be used, which is more suitable for depletion of important or small samples. Albumin depletion was variable for the samples run through this kit (from 11.6 to 83.5% albumin depleted), with more albumin being depleted from plasma samples than from IF samples. There was also some loss of proteins of interest in the plasma samples following albumin depletion with this kit.

The final kit chosen was the Qiagen Qproteome^{TM} Murine Albumin Depletion Kit, which uses affinity columns with monoclonal antibodies that bind albumins. This kit was also quick and easy to use, although used spin down columns. PBS was used as the dilution buffer, which is regularly used in the laboratory so extra time was not required for buffer preparation. The protocol required only a small sample volume (25µL) and also had the option for additional wash steps to increase the recovery of proteins following albumin depletion. The kit is also specific for rat and mouse samples so should be better at depleting albumin from the samples than the other kits tested. Again, there was some variability between albumin depletion across the samples (from 19.4 to 79.5% albumin depleted) but it provided the most consistent reduction in albumin across the samples tested compared to the other two kits. Analysis of the four proteins of interest suggested some loss of proteins in the plasma samples following albumin depletion. However, columns with antibodies to albumin have been previously reported to have the least non-specific binding (Bjorhall et al., 2005). The methods used in this study would need to be improved as described above in order to support this hypothesis.

Overall, this brief albumin depletion study shows some variability in the amount of albumin removed from samples with all kits, although they all seem to have removed a greater percentage of albumin from the plasma samples than from the IF samples. From these analyses, the Calbiochem and Qiagen kits appear to be better at depletion of albumin without reducing the amount of other proteins in the samples, compared to the BioRad kit (Table 8-1). This agrees with published results and the BioRad kit instructions suggesting that the Cibacron dye method does allow some non-specific binding. Removal of albumin would be advantageous if the protein profile of different samples needed to be compared, especially if proteins of interest are around the same size as albumin (64kDa). Each of the kits used in this study would need to be optimised (number of wash steps, dilution buffer, sample volume) for use with the specific samples required. Further methods of albumin depletion could also be investigated, such as the TCA/acetone precipitation method (Chen et al., 2005).

Table 8-1 Comparison of Albumin Depletion Kits.

Kit	BioRad Aurum™ Affi-Gel® Blue	Calbiochem ProteoExtract®	Qiagen Qproteome™
Method	Cibacron blue dye on bead, cross linked agarose gel, spin down columns	Affinity columns (novel albumin-specific resin), gravity flow columns	Affinity columns (immobilised monoclonal antibodies), spin down columns
Sample volume required	75-125µL (75µL)	20-60µL (35µL)	25µL
Buffer	20mM Tris buffer, pH8.3	Albumin binding buffer supplied with kit	PBS
Number of wash steps	1	2	3 + 2 optional
Time	Fast	Fast	Fast
Ease of use	Easy to use although had to make up buffer	Easy to use – buffer supplied with kit	Easy to use
Effectiveness (% albumin depleted)	0.2-87.6%	11.6-83.5%	19.4-79.5%
Problems	Hard to tell whether resin was overdry during centrifugation step	none	none

Following results previously described in this thesis, suggesting that proteins only leak from STs into IF following damage to the BTB, and therefore suggesting that a general biomarker for early detection of testicular toxicity cannot be found, an alternative method for detection of testicular toxicity was investigated, namely automated immunohistochemistry and image analysis. Although this would not provide an early warning detection system for testicular toxicity, it may improve the detection of testicular damage at the end of a toxicology study.

One germ cell protein, DAZL, was investigated with a limited number of testis sections from the $CdCl_2$, MAA and DNB studies carried out previously. The automated immunohistochemistry method was quicker than the usual 'hands on'

approach and should produce more consistent results. Staining with an antibody to DAZL was successful, and the results from the image analysis showed that a significant reduction in the percentage of DAZL staining in each tissue section could be detected in the high dose groups of all three toxicant treatments. The limitations to this investigative study were the number of sections used (3 or 4/dose group) and if more had been used, a difference may have been detected in the lower dose groups. On the other hand, observation of a significant decrease in the high dose groups with such a small number of samples also suggests that the technique is sensitive to testicular damage.

This method could be useful in routine toxicology testing, as it provides a relatively simple quantification of something that is normally only descriptive. It could also allow for quick detection of some testicular damage, which could then be further investigated without a pathologist having to look closely at lots of tissue sections. This has cost implications as it could be set up as a high-throughput screen carried out by a less qualified person.

A limitation of the investigative study described here, is that only one marker was used so only one area of testicular/germ cell damage was assessed. Further markers targeting different stages of germ cells, such as VASA or FABP9, or different areas of the testis, such as Sertoli cell or Leydig cell markers, could also be investigated and a panel of markers set up for a high-throughput screen, which would be more sensitive at detecting damage as well as providing more information about the damage caused. Different markers could be selected depending on the potential target of the drug compound being investigated. Of course, this would involve more tissue sections and more immunohistochemistry runs, but the system used for this investigation was relatively fast and easy to carry out so a screening system could easily be set up.

9 General Discussion

9.1 Objective and Approach

The objective of this project was to investigate whether sensitive biomarkers can be identified and measured as a means of providing an 'early warning' of testicular damage in non-clinical toxicology studies. Biomarkers would aid the detection of testicular toxicity in such studies by providing information about testicular damage before necropsy at the end of a study, and providing an indication of how any testicular damage may progress throughout the study, compared to the current method of detection of testicular toxicity which is based mainly on histopathology at the end of the study. Inhibin B, ABP, LDH-C4, and creatine, amongst other molecules, have previously been investigated as biomarkers for testicular toxicity (Section 2.5.2.7). However, all of these proved to have limitations and have not been accepted for widespread use in the pharmaceutical industry or for monitoring adverse effects in the clinic.

The approaches taken to investigate the potential use of biomarkers to detect testicular toxicity were based on results described by Turner et al., 1996, who showed preliminary evidence that disruption of spermatogenesis by heat treatment, increased the number and amounts of seminiferous tubule-derived proteins in testicular interstitial fluid, and suggested that such proteins therefore have potential to be markers of disruption to spermatogenesis. This promoted the hypothesis that germ cell derived proteins may leak from the seminiferous tubules into the interstitial fluid, and then into the bloodstream, following toxicological insult to the testis, which could be due to either germ cell damage or loss of integrity of the blood-testis barrier. To investigate this hypothesis, the approach was to establish whether proteins do leak from STs following testicular toxicity, and investigate the conditions leading to such leakage. This involved the administration of three well-studied testicular toxicants to adult male rats, which specifically target different components of

seminiferous tubules, followed by an evaluation of the damage caused and analysis of proteins present in the interstitial fluid.

For these studies three related techniques were used to investigate potential protein biomarkers. First, an antibody to seminiferous tubule conditioned medium (STCM antibody), prepared by a previous PhD student (Turner, 1996) was used to investigate a range of seminiferous tubule derived proteins in tissue and interstitial fluid samples collected from the toxicant studies. Second, 1D gels stained with GelCode, a Coomassie dye based stain, were used to investigate the total protein composition in interstitial fluid samples. Third, specific proteins were selected from an Affymetrix Genechip® screen carried out at GSK, based on the criteria for a specific protein biomarker for testicular toxicity. These proteins were investigated in samples collected from the toxicant studies, and allowed investigation of a second hypothesis, namely that smaller molecular weight proteins were thought to be more likely to leak out of seminiferous tubules into IF.

9.2 Protein Leakage

The model testicular toxicants, cadmium chloride (which disrupts the blood-testis barrier and spermatogenesis), methoxyacetic acid (which causes degeneration of pachytene spermatocytes) and 1,3-dinitrobenzene (which causes Sertoli cell vacuolation) were administered to adult male rats at doses chosen to produce mild and moderate toxicological effects on the testis. To investigate whether proteins leaked from STs into IF following toxicant treatment, the proteins present in IF samples from control and treated animals were compared by Western blotting with the STCM antibody and Coomassie gel analysis. Testicular damage was assessed by immunohistochemistry with the STCM antibody and an evaluation of the blood-testis barrier utilised immunofluorescent analysis of tight and adherens junction proteins, together with functional analysis using the biotin tracer technique.

The results from the toxicant studies showed that proteins did not leak from the STs into IF following treatment with any dose of either MAA or DNB, even though the pachytene spermatocytes were depleted, or Sertoli cell vacuolation occurred following the respective treatment. Neither of these treatments were shown to have an effect on the integrity of the BTB either. Leakage of some ST proteins was observed following treatment with high doses of cadmium chloride. A loss of integrity of the BTB was also observed following treatment with high doses of cadmium chloride. These results suggested that a loss of integrity of the blood-testis barrier is required for leakage of proteins from STs into IF.

To further investigate these results, three further approaches were taken to specifically target the BTB to elucidate whether loss of BTB integrity really is the factor leading to leakage of proteins from STs into IF following cadmium chloride treatment. A physiological approach was taken involving study of the development of the blood-testis barrier in young rats. The BTB forms between postnatal days 15-19 in the rat (Vitale et al., 1973; Russell et al., 1989), so it was hypothesised that germ cell derived proteins may be present in IF before the BTB is formed but not after, due to the presence of the barrier preventing them from leaking out. Four ages were investigated across the BTB development period (postnatal days 10, 15, 18, 25) and the BTB was shown to be present at day 25, forming at day 18, and absent at days 10 and 15, in agreement with the literature. Analysis of the proteins present in IF collected from rats at each age, however, highlighted a limitation of this approach, that many (germ cell) proteins of interest are not expressed until later in the development process/in later stage germ cells, and therefore cannot be used to investigate this hypothesis. One germ cell protein, DAZL, that was shown to be expressed in spermatocytes from day 10, was investigated by Western blotting in IF samples from the different aged rats. The results suggested no differences in the amount of DAZL present in IF samples across the age range. However, whether the protein band analysed on the blots was actually DAZL protein was not clear. The development studies, therefore, proved inconclusive in investigating proteins in IF before and after the BTB had formed.

The two further approaches to elucidate the role for loss of integrity of the BTB in leakage of proteins from STs into IF, involved specifically targeting junction proteins in the BTB. Intra-testicular administration of glycerol was shown by Wiebe et al., 2000, to cause disruption of occludin in the BTB, and intra-testicular administration of TGF-β3 was validated as a model for BTB disruption by Xia et al., 2008. Adult male rats were administered either glycerol or TGF-β3, and tissue/IF samples collected 48 hours later to investigate whether specific disruption of the BTB led to leakage of proteins from STs into IF. Treatment with glycerol caused a localised disruption to the organisation of the STs (Sertoli cell vacuolation and germ cell disruption), with loss of occludin protein from the site of the BTB in some STs. This disruption was thought to be localised to the site of injection although this could not be confirmed. Analysis of the proteins present in IF samples following glycerol treatment suggested a trend for an increase in the amount of some proteins compared to IF from control testes, suggesting that some proteins are leaking from tubules affected by the glycerol treatment. Further investigations could be carried out to provide more concrete evidence for this result, by using an increased number of samples, or leaving a longer period of time between administration of glycerol treatment and collection/analysis of samples, based on observations in the literature where effects on spermatogenesis were observed 1 week following administration of glycerol treatment (Igdoura and Wiebe, 1994).

Treatment with TGF-β3 also caused some localised damage to a small number of seminiferous tubules. Disruption of occludin from the site of the BTB was observed in some STs, although investigations with the biotin tracer suggested that the BTB was functional following TGF-β3 treatment. Analysis of the proteins present in IF samples following TGF-β3 treatment suggested some proteins could be leaking from STs into IF, however, the results were variable and analysis of a larger number of samples would be required to obtain more reliable results. Nevertheless, the results suggested that only minor leakage of germ cell proteins occurred.

The investigations to elucidate a role for loss of integrity of the BTB and leakage of proteins from STs into IF by specifically targeting the junctions in the BTB with glycerol or TGF-β3 treatment supported the hypothesis that proteins only leak from STs into IF following loss of integrity of the BTB. However, further experiments with these treatments could be carried out, possibly leaving the damage to develop for longer before collecting samples, to try and obtain wider disruption of the BTB throughout the testis, and more significant protein leakage data. To further investigate the effect of BTB disruption on protein leakage, additional treatments to specifically target the BTB, such as intra-testicular injection of TNF-α (Li et al., 2006), or administration of an occludin peptide (Chung et al., 2001), could also be investigated, although these may prove to be limited for the same reasons as the glycerol and TGF-β3 studies (i.e. only localised damage).

9.3 Protein Biomarkers

While investigating whether proteins do leak from seminiferous tubules into interstitial fluid, five potential biomarker protein candidates were selected and evaluated alongside the treatment studies. The proteins investigated were selected from a list of testis specific proteins based on results from a testicular gene analysis screen carried out at GSK, and well-studied germ cell proteins, based on the criteria described in Table 7-1. The selected proteins allowed the investigation of the hypothesis that smaller molecular weight proteins are more likely to leak out of STs than large molecular weight proteins. Samples collected from the toxicant studies were used to evaluate the potential for each biomarker candidate protein.

DAZL and VASA are both well-studied germ cell proteins. DAZL was specifically expressed in spermatocytes, and immunohistochemistry suggested a dose-dependent decrease in DAZL protein following MAA and DNB treatments. However, analysis of DAZL protein in interstitial fluid samples suggested that DAZL does not leak out of STs into IF following high dose $CdCl_2$ treatment or MAA or DNB treatment. There was some discrepancy in the protein bands observed when Western blotting

with the DAZL antibody compared to the predicted size of the protein, so further investigations, such as trying a different antibody, different Western blot system, or analysis of the protein band identified, could be carried out to clarify the discrepancy. However, if DAZL does not leak out of STs into IF following BTB damage, it could not be used as a biomarker. Results obtained with the VASA protein proved more positive. Again a dose-dependent reduction in VASA protein expression in seminiferous tubules was suggested following treatment with $CdCl_2$, MAA and DNB, by immunohistochemical analysis. Evaluation of VASA in IF samples, by Western blotting, suggested that VASA protein was leaking from STs into IF following mid and high dose cadmium chloride treatment in a dose dependent manner. No leakage of VASA was observed following MAA or DNB treatment, further supporting the hypothesis discussed in section 9.2. Surprisingly, VASA is a relatively large protein (76kDa) and perhaps would have been thought to be less likely to leak from STs than DAZL (33kDa).

ADAM3 and calpastatin were selected from the GSK screen. Both proteins were detected in testis sections by immunohistochemistry and both were shown to be localised to spermatids. Analysis of the proteins in IF samples by Western blotting proved unsuccessful with antibodies to both proteins and no conclusions could be drawn. Alternative antibodies for each protein could be sourced in order to try further investigations by Western blotting.

The final biomarker candidate investigated was FABP9, a small protein expressed in spermatocytes and spermatids. The presence of FABP9 was investigated in IF samples from the toxicant studies by Western blotting and FABP9 protein was detected in IF samples from animals treated with mid and high dose $CdCl_2$ in a dose dependent manner. No significant increases in the amount of FABP9 in IF following treatment with either dose of MAA or DNB were observed, again supporting the results discussed in section 9.2. Further investigations with FABP9 were carried out using tissue collected from the glycerol, TGF-β3 and testis development studies. A significant increase in FABP9 protein was detected in IF samples from glycerol

treated testes compared to control, even though the glycerol treatment had only a localised effect on the testis. This result suggests that FABP9 could be a sensitive marker and detect slight disruption to the BTB. No differences were observed in IF samples following TGF-β3 treatment, however, this could be due to the very small amount of damage observed. Results from the development study showed that FABP9 is present only from around day 18, again illustrating the limitation of the physiological approach to investigating the role for the BTB in leakage of proteins from STs.

The protein biomarker investigations suggested that VASA and FABP9 are promising biomarker candidates for detection of damage to the BTB. If they were to be developed for use in regulatory toxicology studies, a sensitive assay would need to be developed, and the potential for detecting changes in the levels of the biomarker in blood samples evaluated. However, these proteins would only be of use in the detection of testicular damage that involved disruption to the blood-testis barrier, rather than general disruption to spermatogenesis. Therefore, such a marker is unlikely to aid detection of testicular toxicity in regulatory toxicology studies.

9.4 Refinements for Detection of Testicular Toxicity

The results obtained in the studies discussed so far suggest that the later initial aims of the project (to identify protein biomarkers that have the potential to signal an adverse change to spermatogenesis after toxic insult and develop a highly-sensitive assay for detection of low-levels of the biomarker) cannot be accomplished. However, improvements to the current detection methods for testicular toxicity and the methods used in these investigations can be discussed.

One problem with the protein analysis throughout this thesis was the large amount of albumin present in the IF samples which limited the amount of protein that could be loaded onto the gels, and therefore the sensitivity to detect low abundance proteins; this would prove to be an even bigger limitation when trying to detect germ cell

proteins in blood plasma. Three commercially available kits were investigated as a potential refinement for analysis of such samples in future studies. The brief investigation using the kits suggested that it was possible to deplete the amount of albumin present in plasma and interstitial fluid samples, although the results were variable with each kit/sample. If an albumin removal kit were to be used in future studies, it would need to be specifically optimised for the samples involved.

An alternative method to aid detection of testicular toxicity was also investigated. Automated immunohistochemistry and image analysis could improve the detection of testicular damage at the end of a regulatory toxicology study, even though they would not be able to provide an early warning detection system. Automated immunohistochemistry with DAZL antibody was carried out using testis sections obtained during the toxicant studies. This method was quicker than the 'hands on' immunohistochemistry methods, and should produce more consistent results. A significant decrease in the percentage of DAZL staining on each testis section was observed following high dose treatment with MAA, DNB and cadmium chloride. A dose-response was not observed, which could be due to the limited number of samples evaluated. A larger sample size should be used to assess the potential for this technique in detection of mild toxicant induced effects.

This method could be used in routine toxicology tests as it provides a quantitative result for something that is normally descriptive, and could be used as a screen for testicular damage, with any positive results followed up by a pathologist. However, only one marker, DAZL, was used in this study, and therefore only one area of the testis (spermatocytes) assessed. A panel of markers could be investigated that target different stages of germ cells, such as VASA or FABP9, or different areas of the testis, such as Leydig cells or Sertoli cells, and then set up as a high-throughput screen for detecting testicular damage.

9.5 Summary

The development of sensitive biomarkers for testicular toxicity would be a useful addition to the current endpoints used for assessment of testicular toxicity. A variety of markers have been investigated in the past although none have been accepted for widespread use. This project aimed to investigate the potential for biomarkers for testicular toxicity based on the idea that germ cell derived proteins may leak from seminiferous tubules into interstitial fluid following toxicological insult. The results obtained suggest that such a biomarker, for general testicular damage, cannot be found, although markers for disruption of the blood-testis barrier (VASA and FABP9) could be further investigated if a biomarker for such damage would be of value. Therefore, further attention could be focussed on improving current methods of detection of testicular toxicity, such as histopathology, potentially by developing an automated immunohistochemical screening system to assess different areas of the testis to provide quick results that could be followed up in more detail.

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